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Universidad del País Vasco
Euskal Herriko Unibertsitatea

Facultad de Medicina y Odontología
Departamento de Fisiología

TARGETING MITOCHONDRIA AND POST-TRASLATIONAL MODIFICATIONS: NEW PROMISING APPROACHES FOR THE TREATMENT OF LIVER DISEASE

TESIS DOCTORAL

Lucía Barbier Torres

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Directoras de tesis:

Dra. María Luz Martínez Chantar

Dra. Teresa Cardoso Delgado

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ABBREVIATIONS

ABBREVIATIONS

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
ABCD1	ATP binding cassette subfamily D member 1
ACADM	Acyl-CoA dehydrogenase, medium chain
ACADL	Acyl-CoA dehydrogenase, long chain
ACC	Acetyl-CoA carboxylase
ADP	Adenosine-5'-diphosphate
AFP	Alpha-fetoprotein
AIF	Apoptosis inducing factor
ALD	Alcoholic liver disease
ALF	Acute liver failure
ALT	Alanine aminotransferase
ALS	Alagille syndrome
AMA	Anti-mitochondrial antibody
AML12	Alpha mouse liver 12
AMPK	AMP-activated protein kinase
ANT	Adenine nucleotide translocase
AP-1	Activator protein 1
APC	Anaphase-promoting complex
Apaf-1	Apoptotic protease activating factor 1
APAP	Acetaminophen
ApoB	Alipoprotein B
ARE	Antioxidant response element
ASBT	Apical sodium BA transporter
ASK-1	Apoptosis signaling-regulating kinase 1
ASM	Acid soluble metabolite
αSMA	Alpha smooth muscle actin
AST	Aspartate aminotransferase
ATP	Adenosine-5'-triphosphate
AUC	Area under the curve
BA	Bile acid
BACAT	Bile acid:amino acid transferase
BACS	Bile acid:CoA synthase
BAT	Biliary atresia
BCA3	Breast cancer-associated protein 3
BCLC	Barcelona clinic liver cancer
Bcl-2	B-cell lymphoma 2
BDL	Bile-duct ligation
BEC	Biliary epithelial cell
BN-PAGE	Blue native-polyacrylamide gel electrophoresis
BSEP	Bile Salt Export Pump
CAD	Caspase-activated DNase
cAMP	Cyclic adenosine monophosphate
CCL2	C-C Motif Chemokine Ligand 2
CDCA	Chenodeoxycholic acid
CD	Cluster of differentiation
CE	Cholesterol ester
c-FLIP	Cellular FLICE inhibitory protein
ChREBP	Carbohydrate response element-binding protein
CHOP	C/EBP homologous protein
CHX	Cycloheximide
CK19	Cytokeratin 19
CLNA	Cardiolipin
c-Myc	V-Myc avian myelocytomatosis viral oncogene homolog
CRL	Cullin-RING ligase
CSN	COP9 signalosome
CYP	Cytochrome P450
CoQ	Coenzyme Q
CPT1	Carnitine palmitoyltransferase
Col1A1	Collagen type I alpha 1

CXCL1	C-X-C motif chemokine ligand 1
Cyt *c*	Cytochrome *c*
DG	Diacylglycerol
DAMP	Damage-associated molecular pattern
DCA	Deoxycholic acid
DCN1	Defective in cullin neddylation 1
DD	Death domain
DILI	Drug-induced liver injury
DISC	Death inducing signal complex
DNA	Deoxyribonucleic acid
DNL	*De novo* lipogenesis
DNP	2,4-dinitrophenol
DR4/5	Death receptor 4/5
ECAR	Extracellular acidification rate
ECM	Extracellular matrix
eNOS	Endothelial nitric oxide synthase
EGFR1	Epidermal growth factor receptor 1
EGR1	Early growth response 1
Epcam	Epithelial cell adhesion molecule
ETC	Electron transport chain
ER	Endoplasmic reticulum
FA	Fatty acid
FACS	Fatty acid acyl-CoA synthase
FADD	Fas-associated protein with death domain
$FADH_2$/FAD	Flavin adenine dinucleotide
FASN	Fatty acid synthase
FATP2	Fatty acid transport protein 2
FBP1	Fructose-Bisphosphatase 1
FBS	Fetal bovine serum
FC	Free cholesterol
FCCP	Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone
FFA	Free fatty acid
FGFR4	Fibroblast growth factor receptor 4
FMN	Flavin mononucleotide
FoxO1	Forkhead box O1
FXR	Farsenoid X receptor
G6PDH	Glucose-6-phosphate dehydrogenase
GalN	D-galactosamine N
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCL	Glutamylcystine ligase
GCDCA	Glycochenodeoxycholic acid
GFP	Green fluorescent protein
GGT	Gamma-glutamyl transpeptidase
GLDH	Glutamate dehydrogenase
GLUT	Glucose transporter
GNMT	Glycine N-methyltransferase
GSH/GSSG	Glutathione reduced/oxidized
GSK3	Glycogen synthase kinase 3
GPx	Gluthathione peroxidase
GRd	Gluthathione reductase
HAT	Histone acetylase
HCC	Hepatocellular carcinoma
HDAC	Histone deacetylase
HE	Hepatic encephalopathy
H&E	Hematoxylin & Eosin
HGF	Hepatocyte growth factor
HIF1α	Hypoxia inducible factor 1 alpha
HK	Hexokinase
HuR	Human antigen R
HO-1	Heme oxygenase 1
HSC	Hepatic stellate cell
IAP	Inhibitor of apoptosis protein
ICAM-1	Intercellular adhesion molecule 1

IDH	Isocitrate dehydrogenase
IGF	Insulin-like growth factor
IGFR	Insulin-like growth factor receptor
IHC	Immunohistochemistry
IκB	Inhibitor of kappaB
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IMM	Inner mitochondrial membrane
IMS	Inner mitochondrial space
IP	Immunoprecipitated proteins
IR	Insulin resistance
IRS	Insulin receptor substrate
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KB	Ketone body
KC	Kupffer cell
KGDH	Ketoglutarate dehydrogenase
KO	Knockout
LCA	Litocholic acid
LCFA	Long chain fatty acid
LDH	Lactate dehydrogenase
LKB1	Liver kinase B1
LPS	Lipopolysaccharide
LXR	Liver X receptor
MAPK	Mitogen-activated kinase
MAT1A	Methionine adenosyltransferase 1A
MCDD	Methionine-choline deficient diet
MCJ	Methylation-controlled J-protein
MDH	Malate dehydrogenase
Mdm2	Murine double minute 2 homolog
MDR	Multidrug resistance protein
MEF2	Myocyte Enhancer Factor 2
MMP	Mitochondrial membrane potential
MO25	Mouse protein 25
MPTP	Mitochondrial permeability transition pore
mtDNA	Mitochondrial DNA
mTOR	Mammalian target of rapamycin
mROS	Mitochondrial reactive oxygen species
MRP	Multidrug resistance-associated protein
MUFA	Monounsaturated fatty acid
NAC	*N*-acetylcysteine
NADH/NAD$^+$	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NAE1	Nedd8-activating enzyme 1
NAFLD	Non-alcoholic fatty liver disease
NAPQI	*N*-acetyl-*p*-benzoquinoneimine
NASH	Non-alcoholic steatohepatitis
NAT	N-acetyltransferase
N-CoR	Nuclear receptor co-repressor
NEDD8	Neural Precursor Cell Expressed, Developmentally Down-Regulated 8
NEDP1	NEDD8-Specific Protease 1
NFκB	Nuclear factor kappaB
NK	Natural killer
NL	Normal healthy liver
NO	Nitric oxide
NOX	NADPH oxidase
NRF2	Nuclear Factor, Erythroid 2 Like 2
NTCP	Na^+-taurocholate cotransporting polypeptide
OA	Oleic acid
OATP	Organic anion-transporting polypeptides
OCR	Oxygen consumption rate
OPA1	Optic atrophy 1
OST	Organic-solute transporter

OXPHOS	Oxidative phosphorylation
PAI1	Plasminogen activator inhibitor 1
PAS	Periodic Acid Schiff
PBC	Primary biliary cirrhosis
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PDC	Pyruvate dehydrogenase complex
PDGF	Platelet-derived growth factor
PDH	Pyruvate dehydrogenase
PE	Phosphatidylethanolamine
PEI	Percutaneous ethanol injection
PEMT	Phosphatidylethanolamine N-Methyltransferase
PFKL	Phosphofructokinase, liver type
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1 alpha
PHB1	Prohibitin 1
PI	Phosphatidylinositol
PI3K	Phosphoinositide-3 kinase
PKC	Protein kinase C
PL	Phospholipid
PPAR	Peroxisome proliferator-activated receptor
PS	Phosphatidylserine
PSC	Primary sclerosing cholangitis
PTEN	Phosphatase and tensin homolog
PTM	Post-translational modification
PTMA	Prothymosin alpha
PUFA	Polyunsaturated fatty acid
PUMA	p53 upregulated modulator of apoptosis
PXR	Pregnane X receptor
RASSF	Ras association domain-containing protein
Rb	Retinoblastome
RFA	Radiofrequency ablation
RIP	Receptor interacting protein
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT-qPCR	Quantitative real-time reverse transcription polymerase chain reaction
SAMe	S-adenosylmethionine
SAPK	Stress activated protein kinase
SC	Supercomplexes
SCD1	Stearoyl-CoA desaturase-1
SCF	Skp1-Cullin-F-box complex
SDH	Succinate dehydrogenase
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SFA	Saturated fatty acid
SHP	Small heterodimer partner
siRNA	Small interfering RNA
SIRT	Sirtuin
SMRT	Silencing mediator of retinoic acid and thyroid hormone receptor
SOCS	Suppressor of cytokine signaling
SOD	Superoxide dismutase
SREBP	Sterol regulatory element-binding protein
STAT	Signal transducer and activator of transcription
STK11	Serine/threonine kinase 11
STRAD	STE-20-related adaptor
SULT2A1	Sulfotransferase Family 2A Member 1
TACE	Transcatheter arterial chemoembolization
TCA	Tricarboxylic acid
TCDCA	Taurochenodeoxycholic acid
TFAM	Transcription factor A
TG	Triglyceride
TGFβ	Transforming growth factor beta
TIM23	Translocase of the inner membrane 23

TNFα	Tumor necrosis factor alpha
TNFR	Tumor necrosis factor receptor
TPN	Total parenteral nutrition
TRAIL	TNF-related apoptosis inducing ligand
TSA	Trichostatin A
TUDCA	Tauroursodeoxycholic acid
Ub	Ubiquitin
Ube1	Ub E1 enzyme
UCP	Uncoupling protein
UDCA	Ursodeoxycholic acid
UGT2B4	UDP glucuronosyltransferase 2 family, polypeptide B4
UPS	Ubiquitin-proteasome system
VCAM-1	Vascular cell adhesion molecule 1
VDR	Vitamin D receptor
VEGF	Vascular endothelial growth factor
VHL	Von Hippel-Lindau
VLDL	Very low-density lipoprotein
XIAP	X-linked inhibitor of apoptosis
WT	Wild type

1. RESUMEN/SUMMARY

1. RESUMEN

La enfermedad hepática es una causa muy importante de morbilidad y mortalidad en todo el mundo que afecta tanto a la población adulta como a la infantil. Según datos de la Organización Mundial de la Salud de 2013, alrededor de 29 millones de personas sufren lesiones hepáticas crónicas en Europa, más de 35 millones padecen enfermedades hepáticas en Estados Unidos y cerca de 2 millones de pacientes mueren anualmente por cirrosis y cáncer hepático. Cabe destacar que la incidencia de la enfermedad hepática está aumentando de forma alarmante con los años y representa hoy en día un gran problema de salud global. Se conocen en torno a 100 tipos de enfermedades hepáticas, siendo la hepatitis viral y el consumo excesivo de alcohol las más comunes. Sin embargo, en los últimos años, el hígado graso no alcohólico (HGNA) y la hepatotoxicidad por fármacos han emergido como causas importantes de enfermedad hepática, hecho que se asocia a cambios en nuestro estilo de vida como una mala alimentación, una vida más sedentaria y un consumo excesivo de medicamentos. Además, las alteraciones genéticas y autoinmunes, incluyendo formas colestásicas de hepatitis, siguen siendo importantes causas de lesión hepática. La enfermedad hepática es la situación en la que el hígado, uno de los órganos más grandes e importantes del cuerpo humando, sufre un daño. Este daño, dependiendo de la causa y de si es detectado de forma temprana, puede ser reversible, aunque en la mayoría de los casos, incluyendo el HGNA y las enfermedades colestásicas, se vuelve crónico y evoluciona con el tiempo a cirrosis y carcinoma hepatocelular (CHC), condiciones irreversibles y de no buen pronóstico. Por otro lado, el daño hepático puede ser agudo y llevar a un fallo fulminante que requiera un trasplante urgente, como es el caso del daño hepático por fármacos. El hígado es un órgano metabólicamente muy activo que se encarga de funciones vitales como son la digestión, la detoxificación y limpieza de la sangre y el metabolismo y distribución de nutrientes y energía al resto del cuerpo. Estas funciones hacen que el hígado se encuentre más expuesto que otros órganos a sustancias nocivas y sea por tanto más susceptible a sufrir lesiones. A pesar de la alarmante indecencia de la enfermedad hepática, los mecanismos moleculares implicados en su desarrollo y progresión no han sido del todo elucidados, lo que limita en gran medida la existencia de tratamientos terapéuticos más eficaces que los actualmente disponibles. En la mayoría de los casos la enfermedad es detectada en un estadío avanzado y carece de un tratamiento establecido. Por todo esto, un mejor conocimiento de los mecanismos subyacentes a la enfermedad hepática es crucial para poder desarrollar nuevas estrategias terapéuticas y preventivas más efectivas.

El principal objetivo de este estudio ha sido identificar nuevos mecanismos implicados en la enfermedad hepática en relación a la disfunción mitocondrial y la alteración de rutas de modificaciones post-traduccionales (MPT). Como resultado de la alta actividad metabólica del hígado, los hepatocitos son uno de los tipos celulares con más densidad de mitocondrias y uno de los más susceptibles de sufrir alteraciones en la función mitocondrial. Las mitocondrias se encargan de generar energía para la célula y de controlar el balance energético y la muerte celular y su función puede verse alterada tanto por mutaciones en genes mitocondriales como por sustancias exógenas como pueden ser los virus, el alcohol o los fármacos. En los últimos años el papel de la mitocondria en el daño hepático se ha empezado a considerar como un mecanismo importante y se han detectado alteraciones en su función en enfermedades como HGNA, hepatotoxicidad por fármacos, colestasis y CHC. Por otro lado, las MPTs son mecanismos esenciales para la señalización, el metabolismo y la actividad celular, regulando la función y homeostasis de las proteínas. Dado que las MPTs son esenciales para la fisiología normal del hígado, cabe pensar que alteraciones en sus rutas pueden estar implicadas en la enfermedad hepática. De hecho, cambios en acetilación, ubiquitinación y nedilización han sido descritas en diferentes lesiones como el cáncer hepático y la colestasis.

Para evaluar la implicación de la mitocondria en el desarrollo de la enfermedad hepática, hemos estudiado el papel de la proteína J controlada por metilación (MCJ) y de la prohibitina 1 (PHB1), dos proteínas mitocondriales con papeles opuestos en cuanto a actividad mitocondrial, en diferentes lesiones en las que la función mitocondrial está altamente comprometida, como son el HGNA, al daño hepático por fármacos, las enfermedades colestásicas y el CHC. Para ello hemos usado dos modelos de ratón knockout (KO) de estas proteínas: el ratón MCJ KO, que presenta una activación en la función mitocondrial, y el *Phb1* KO, que presenta disfunción mitocondrial y lesiones hepáticas visibles. Por otro lado, para tratar nuestra otra hipótesis de que la desregulación de MPTs puede estar implicada en la patogenia de la enfermedad hepática, hemos estudiado posibles alteraciones en acetilación y ubiquitinación en el modelo *Phb1* KO, ya que la deficiencia de PHB1 induce cambios en la acetilación de histonas y la actividad del proteasoma. Además, hemos evaluado el impacto de la nedilización en el desarrollo de CHC, centrándonos en descubrir nuevas dianas de esta MPT y evaluar su inhibición como terapia, en base a los resultados previos descritos por nuestro laboratorio donde identificamos la proteína oncogénica de unión a ARN HuR, altamente expresada en cáncer hepático y de colon, diana de nedilización.

De acuerdo con nuestros resultados, MCJ es un regulador negativo del metabolismo mitocondrial en el hígado a diferentes niveles. Su ausencia produce un aumento de la respiración mitocondrial a través de los complejos I y II de la cadena respiratoria mitocondrial y consecuentemente, la aceleración de otras rutas metabólicas como la glicolisis, el ciclo de Krebs y la β-oxidación. Tanto en HGNA como en daño hepático por fármacos, los niveles de MCJ se encuentran altamente inducidos, lo que sugiere un papel para esta proteína en la lesión hepática mitocondrial. De forma

importante, hemos demostrado que la deficiencia en MCJ podría ser una terapia prometedora para estas enfermedades y probablemente para otras en asociadas a daño mitocondrial, ya que su silenciamiento en modelos experimentales ha resultado muy efectivo, manteniendo la función mitocondrial y los niveles de ATP en la célula, bloqueando la muerte celular y atenuando el daño hepático y la progresión de la enfermedad.

Por otro lado, la ausencia de PHB1, previamente asociada a estrés oxidativo, a la predisposición al daño hepático y al desarrollo de fibrosis y CHC, tiene también un papel importante en colestasis. Hemos visto que su expresión se encuentra disminuida específicamente en pacientes con enfermedades colestásicas y que su deficiencia predispone al daño por colestasis en modelos experimentales. Tanto a nivel mitocondrial como a nivel transcripcional a través de la histona deacetilasa 4 (HDAC4), la deficiencia de PHB1 está ligada a una mayor muerte celular por ácidos biliares. Hemos observado que PHB1, a parte de ser esencial para la respiración mitocondrial, es esencial para el correcto funcionamiento del proteasoma en el hígado y para la homeostasis y localización subcelular de HDAC4. En este sentido, hemos observado que la disminución de la expresión de HDAC4 mejora el daño hepático en los ratones *Phb1* KO y podría ser por tanto una diana terapéutica para enfermedades colestásicas asociadas a bajos niveles de PHB1.

Por último, gracias al modelo *Phb1* KO que simula perfectamente la enfermedad hepática desde la respuesta inflamatoria hasta el desarrollo de CHC, hemos presentado evidencias sobre el importante papel de la nedilización en la carcinogénesis hepática. Hemos observado que esta MPT estabiliza LKB1 y Akt en el hígado, siendo por tanto el mecanismo de sobreexpresión de estas quinasas altamente implicadas en metabolismo y cáncer, confiriendo a los hepatocitos ventajas metabólicas y de supervivencia que promueven su transformación maligna. Estas observaciones han sido validadas en pacientes con CHC donde hemos observado que tanto la nedilización como los niveles de LKB1 y Akt están muy inducidos, correlacionándose con la malignidad de la enfermedad. Tanto a nivel de proteína como de expresión génica, la nedilización se encuentra más aumentada en pacientes con peor pronóstico y se ha asociado con una menor supervivencia. En cuanto a inhibir la nedilización como terapia para el CHC, el tratamiento con el fármaco inhibidor de nedilización MLN4924 en ratones *Phb1* KO, ha mostrado una alta efectividad anti-tumoral, induciendo la muerte de las células cancerosas y mejorando al mismo tiempo el estado del hígado. Reforzando este resultado, el silenciamiento de Nedd8 en el modelo xenograft de CHC con células humanas de hepatoma HepG2 bloqueó la progresión de los tumores. En ambos casos la regresión de los tumores se ha asociado a cambios metabólicos dependientes de LKB1 y Akt que inducen la muerte de las células cancerosas. Respecto a esto, hemos observado que la muerte celular por inhibición de la nedilización está mediada, en parte, por la desestabilización de LKB1, Akt y HuR, ya que altos niveles de estas proteínas contrarrestan por completo su efecto anti-tumoral. Dada la complejidad del CHC en cuanto a alteración de rutas de señalización, el haber descubierto que la nedilización, que es un mecanismo de regulación global de proteínas, esté implicado en su patogenia supone un avance importante que podría mejorar notablemente su tratamiento y pronóstico.

En conclusión, nuestros resultados corroboran que tanto una correcta función mitocondrial como la homeostasis de proteínas son fundamentales para el funcionamiento normal del hígado y que alteraciones en ambos procesos están asociados con la lesión hepática. Además, el descubrimiento de nuevos mecanismos implicados en la enfermedad hepática, como son la desregulación de MCJ y PHB1, a nivel mitocondrial, y aberraciones en las rutas de ubiquitinización, acetilación y nedilización, en cuanto a MPTs, abren nuevas vías para el desarrollo de nuevas terapias para el tratamiento de diferentes enfermedades de hígado.

1. SUMMARY

Liver disease is an important cause of morbidity and mortality worldwide affecting both adults and children. According to the World Health Organization data from 2013, about 29 million people suffer from chronic liver injury in Europe, more that 35 million suffer from liver disease in the United States and about 2 million patients die annually from cirrhosis and liver cancer. It should be noted that the incidence of liver disease is increasing alarmingly over the years and is now considered a global health problem. There exist around 100 types of liver disease, being viral hepatitis infections and alcohol abuse the most common. However, in the last few years non-alcoholic fatty liver disease (NAFLD) and drug-induced liver injury (DILI) have emerged as major causes of liver disease, a fact that is associated to changes in our lifestyle like bad dietary habits, a more sedentary life and excessive drug consumption. Additionally, genetic and autoimmune alterations including cholestatic disorders are still important causes of hepatic injury. Liver disease is the situation in which the liver, one of the largest and most important organs of human body, is damaged. This damage, depending on the cause and whether it is detected early may be reversible, although in most cases including NAFLD and cholestatic liver diseases it chronifies and progresses over time to cirrhosis and hepatocellular carcinoma (HCC), irreversible condition with not very good prognosis. On the other hand, liver damage can be acute and lead to fulminant liver failure requiring an urgent transplant, as is the case of DILI. The liver is a metabolically very active organ that is responsible for vital functions such as digestion, blood detoxification and clearance and metabolism and distribution of nutrients and energy to the rest of the body. All these functions make the liver more exposed to harmful substances than other organs and hence, more vulnerable to injury. Despite the alarming incidence of liver disease, the molecular mechanisms involved in its development and progression have not been fully elucidated, which largely limits the availability of more effective therapeutic treatments. Furthermore, in most cases the disease is detected at an advanced stage and lacks an established treatment. For all these reasons, a better understanding of the mechanisms underlying liver disease is crucial for the development of more effective therapeutic and preventive strategies.

The main objective of this study was to identify new mechanisms involved in liver disease in relation to mitochondrial dysfunction and the alteration of post-translational modification (PTM) pathways. As a result of the tremendous metabolic activity of the liver, hepatocytes are one of the cell types with the highest density of mitochondria and one of the most susceptible to suffer alterations in mitochondrial function. Mitochondria are in charge of generating energy for the cell and controlling energy balance and cell death, and their function may be altered by mutations in mitochondrial genes as well as by exogenous substances such as viruses, alcohol or drugs. In the last few years the role of mitochondria in liver injury has begun to be considered as a major mechanism and moreover, alterations in their function have been detected in diseases like NAFLD, DILI, cholestasis and HCC. On the other hand, PTMs are essential mechanisms for signaling, metabolism and cellular activity, regulating the function and homeostasis of proteins. Since PTMs are necessary for normal liver physiology, alterations in their pathways may be involved in liver disease. Indeed, aberrant acetylation, ubiquitination and neddylation have been already described in different disorders such as liver cancer and cholestasis.

In order to evaluate the involvement of mitochondria in the pathogenesis of liver disease, we have studied the role of methylation-controlled J-protein (MCJ) and prohibitin 1 (PHB1), two mitochondrial proteins with opposite roles in mitochondrial function, in different disorders in which mitochondrial function is highly compromised like NAFLD, DILI, cholestasis and HCC. For this purpose we have used two knockout (KO) mouse models for these proteins: the MCJ KO mouse, which presents activation of mitochondrial function, and *Phb1* KO, which presents mitochondrial dysfunction and visible liver lesions. To discuss our other hypothesis that deregulation of PTMs may be implicated in the pathogenesis of liver disease, we have studied possible alterations in acetylation and ubiquitination in the *Phb1* KO model, since it is known that PHB1 deficiency induces changes in histone acetylation and proteasome activity. Furthermore, we have evaluated the impact of neddylation in HCC development focusing on discovering new targets of this PTM and evaluating its inhibition as a therapeutic approach, based on the previous results described by our laboratory where we identified the oncogenic RNA binding protein HuR, highly expressed in liver and colon cancer, as a neddylation target.

According to our results, MCJ is a negative regulator of mitochondrial metabolism in the liver at different levels. Its absence results in an increase of mitochondrial respiration through complexes I and II of mitochondrial respiratory chain and consequently, in the acceleration of other metabolic pathways such as glycolysis, the TCA cycle and β-oxidation. In both NAFLD and DILI, MCJ expression levels are significantly induced, suggesting a role for this protein in mitochondrial liver injury. Notably, we have demonstrated that MCJ deficiency could be a promising therapy for these diseases, and probably for others in which there is mitochondrial damage, since its silencing in experimental models has been very effective in maintaining mitochondrial function and ATP levels in the cell, blocking cell death and attenuating liver damage and the progression of the disease.

On the other hand, the absence of PHB1, which is associated with oxidative stress and is known to predispose

to liver damage and the development of fibrosis and HCC, also plays an important role in cholestasis. We have seen that its expression is reduced specifically in patients with cholestatic diseases and that its deficiency predisposes to cholestatic liver injury in experimental models. Both at mitochondrial and transcriptional level through histone deacetylase 4 (HDAC4), PHB1 deficiency is linked to increased bile acid-induced cell death. We have observed that PHB1, apart from being essential for mitochondrial respiration, is essential for the proper function of the proteasome in the liver and for the homeostasis and subcellular localization of HDAC4. In this regard, we have observed that diminishing the expression of HDAC4 reduces liver damage in *Phb1* KO mice and could therefore be a therapeutic target for cholestatic diseases associated with low levels of PHB1.

Finally, thanks to the *Phb1* KO model that mimics perfectly liver disease from the inflammatory response to the development of HCC, we have presented evidence of the important role of neddylation in hepatic carcinogenesis. We have observed that this PTM stabilizes LKB1 and Akt in the liver, thus being the mechanism of overexpression of these kinases highly involved in metabolism and cancer, conferring to the hepatocytes metabolic and survival advantages that promote their malignant transformation. These findings have been validated in patients with HCC where we have observed that both neddylation and the levels of LKB1 and Akt are highly induced, correlating with the malignancy of the disease. At both the protein and gene expression levels, neddylation is more increased in patients with poorer prognosis and has been associated with lower survival. Regarding the inhibition of the neddylation pathway as a therapy for HCC, the treatment with the drug MLN4924, inhibitor of neddylation, in *Phb1* KO mice has shown high anti-tumor effectiveness, inducing death in cancer cells and improving liver status at the same time. Reinforcing this result, the silencing of Nedd8 in the HCC xenograft model of human hepatoma HepG2 cells blocked the progression of tumors. In both cases the regression of the tumors was associated with metabolic changes dependent on LKB1 and Akt that induce the death of cancer cells. In this regard we have observed that cell death induced by the inhibition of neddylation is mediated, in part, by the destabilization of LKB1, Akt and HuR, since high levels of these proteins completely counteract its anti-tumoral effects. Given the complexity of HCC in terms of altered signaling pathways, having unraveled that neddylation, which is a mechanism of global protein regulation, is implicated in its pathogenesis is an important breakthrough that could improve considerably its treatment and prognosis.

In conclusion, our results corroborate that both a correct mitochondrial function and protein homeostasis are crucial for the proper function of the liver and that alterations in both processes are associated with liver injury. In addition, the discovery of new mechanisms involved in liver disease, such as the deregulation of MCJ and PHB1, at the mitochondrial level, and aberrations in the ubiquitination, acetylation and neddylation pathways, in terms of PTMs, open new avenues for the development of novel therapies for the treatment of different liver diseases.

2. INTRODUCTION

2. INTRODUCTION

2.1 LIVER PATHOLOGY

The liver is the largest solid and most metabolically complex organ in the human body[1,2]. It is involved in major vital functions such as digestion, detoxification, metabolism, immunity and nutrient storage and performs many essential biological processes including glucose homeostasis, synthesis of fatty acids (FAs), amino acids and blood proteins, production of bile and clearance of the blood from harmful substances[2,3,4]. All these functions make the liver an essential organ providing energy and nutrients to the rest of the body[3,5]. However, due to its central role in metabolism, the liver is exposed to a large amount of toxicants and is more susceptible to injury and dysfunction that other organs[6]. Hence, it is not surprising that it is an organ with capacity for natural regeneration[7].

Although liver disease is stereotypically linked to alcohol or viral infections, there are over 100 forms of liver disease caused by a variety of factors affecting everyone from infants to older adults[8]. Furthermore, liver diseases can be acute with fulminant liver failure or chronic and evolve over time to cirrhosis and liver cancer[9-11]. Genetic and autoimmune alterations are important causes of cholestatic liver injury and liver failure, and mostly affect children[12]. Also, changes in the way of life like bad dietary habits (obesity and diabetes)[13] or excessive drug consumption[14] have become major risk factors for liver disease. Indeed, the prevalence and mortality rates of liver disease have been increasing for the past 20 years and represent a global health problem[15,16]. According to the World Health Organization 29 million people in Europe suffer from chronic liver injury, 35 million in the United States have liver disease and about 2 million patients die annually from cirrhosis and liver cancer.

Mitochondrial alterations have been documented in a variety of liver diseases and have been proposed as a common mechanism in their pathogenesis[17]. For this reason, the role of mitochondria is being deeply investigated in both acute and chronic human liver diseases. On the other hand, deregulation of post-translational modifications (PTMs) has also been observed in a variety of liver diseases[18,19] and its involvement in the pathogenesis of liver disease is also being profoundly investigated.

In this first part, a brief overview of major liver diseases associated with mitochondrial dysfunction as are drug-induce liver injury (DILI), non-alcoholic fatty liver disease (NAFLD), cholestatic liver diseases and hepatocellular carcinoma (HCC), will be provided.

2.1.1 Drug-induced liver injury (DILI)

The liver plays a central role in drug metabolism and clearance and is therefore susceptible to DILI. The main cause of DILI development is the abusive intake of drugs. However, there are evidences supporting the role of other factors such as sex, age and chronic liver disease in its pathogenesis[20]. DILI is a leading cause of acute liver failure (ALF) and transplantation in the United States and most of Europe[21,22]. It has an estimated annual incidence between 10-15 cases per 100,000 patients[23].

Acetaminophen (APAP), also known as paracetamol, is the drug most frequently involved in DILI, representing over 50% of cases of ALF in adults in the United States[24]. Hence, the hepatic metabolism of APAP has been broadly studied and is used as example of the toxicity of most of the drugs. About 10% of APAP is metabolized by the enzyme CYP2E1 (cytochrome P450) to the toxic metabolite *N*-acetyl-*p*-benzoquinoneimine (NAQPI), which in normal circumstances conjugates to glutathione (GSH), the main antioxidant of the cell, to be detoxified and excreted into urine[23]. In cases of APAP overdose, GSH is depleted and NAPQI, which is highly reactive, covalently binds to intracellular and mitochondrial proteins disrupting their structure and function and leading to hepatocyte death and liver failure[25]. Moreover, idiosyncratic drugs cause around 10% of the clinical cases of DILI[26], including several forms of cholestatic liver injury, which can present acutely or in the form of chronic liver disease[27]. Whereas drug-induced steatosis is a common event in DILI patients, only a small proportion of patients may develop chronic liver disease[28]. Early recognition of drug-induced liver reactions is essential to minimize injury.

2.1.2 Cirrhosis

Cirrhosis is a complication at the terminal stages of chronic liver disease that involves the irreversible scarring of the liver and poor liver function. As cirrhosis progresses and the amount of scar tissue and regenerative nodules in the liver increases, the liver loses its ability to function[11]. Cirrhosis is a leading cause of illness and death worldwide (1 million deaths annually) and can progress to a number of complications, including liver cancer and liver failure[16]. Depending on the stage of the disease at the time of the diagnosis (decompensation, gastrointestinal bleeding, varices and ascitis), its mortality can range between 1 and 57%[29]. Although recent studies have proposed the reversibility of cirrhosis[30], the current management of the disease focuses on prevention and early intervention to slow the progression of liver injury and reduce the risk of further complications. If the disease progresses to liver failure, patient will require liver transplantation[11].

Alcohol abuse and viral hepatitis B and C, diseases that are not on the scope of this thesis, are the most common causes of liver cirrhosis, although there are other important causes with increasing prevalence, such as NAFLD and cholestatic liver diseases[11-13].

2.1.2.1 Non-alcoholic fatty liver disease (NAFLD)

NAFLD is the most common chronic liver disease in Western world and is becoming a major health problem worldwide[31,32]. It is associated to **obesity**, **type 2 diabetes** and **metabolic syndrome**, disorders with increasing prevalence in the general population[13,33].

NAFLD comprises a spectrum of clinical and histopathological disorders ranging from accumulation of lipids within hepatocytes (hepatic steatosis) to hepatic steatosis with inflammation (steatohepatitis, also known as non-alcoholic steatohepatitis or NASH), fibrosis, cirrhosis and ultimately HCC[13,34]. The accumulation of lipids in the liver results from combined circulating free FAs (FFA), synthesis *de novo* and fats from the diet[35]. When steatosis is accompanied by features of necroinflammation and hepatocyte ballooning, the diagnosis of NASH can be made[36].

Although steatosis is associated to a good (reversible) prognosis, around 20% of NASH patients may develop cirrhosis within 10 years[37,38] and are at risk of developing liver failure and HCC (4-27%)[39]. Importantly, NAFLD is the third most common cause for liver transplantation with 30% prevalence in the United States[40]. Hence, NAFLD represents a rising threat to public health[32,41].

2.1.2.2 Cholestatic liver diseases

Cholestasis literally means "a standing still of bile". Even though cholestatic liver diseases represent a heterogeneous group of disorders, all of them are characterized by an impairment of bile flow from the liver to the intestine that leads to the retention and accumulation of hydrophobic bile acids (BAs) in the liver[42,43]. This accumulation of bile within the liver causes hepatocyte damage, inflammation and liver complications such as liver failure and cirrhosis[44,45].

The two basic subtypes of cholestasis are **obstructive** and **metabolic** cholestasis[46]. While obstructive cholestasis is caused by a mechanical blockage in the ductular system, metabolic cholestasis is caused by disturbances in bile formation. Notably, cholestatic liver diseases can be caused by different factors such as inflammation, viral infections, pancreatic, liver and biliary tree tumors, pregnancy and also by **genetic and autoimmune** disorders[47–53]. Moreover, it has been shown that drugs can also cause cholestatic liver injury, from mixed hepatocellular cholestatic injury to impairement of canalicular bile flow or obstructive cholestasis due to injury to bile duct epithelium[27]. Although cholestasis is not a primary cause of death, it is a cause of considerable morbidity affecting people of every age[12].

In adults, **primary biliary cirrhosis** (PBC) and primary sclerosing cholangitis (PSC) are the most common chronic cholestasis diseases[12]. Secondary biliary cirrhosis is also common and occurs when there is a prolonged mechanical obstruction, PSC, which is a disease of the bile ducts, or other diseases that promote cholestasis, such as **biliary atresia** (BAT) and cystic fibrosis. In children, BAT and inherited syndromes of intrahepatic cholestasis (e.g **Alagille syndrome** or ALS) are the most common causes of chronic liver disease[54,55].

PBC is a chronic liver disease that leads to progressive cholestasis. The etiology of PBC is unclear although it is widely thought to be an autoimmune disease triggered by a combination of genetic and environmental factors. Indeed, PBC is characterized by the presence of anti-mitochondrial antibodies (AMAs) and the destruction of bile ducts[56,57]. BAT is a common childhood disease that may also affect adults, primarily women, where bile ducts are abnormally narrow, blocked or even absent. Although the causes of BAT are not well understood, it has been associated to genetic defects, autoimmune and inflammatory responses and to viral infections and toxins[54]. ALS is caused by mutations in the genes *JAG1* (90% of cases) and *NOTCH2* inherited from one parent, which cause defects in the development and the formation of bile ducts[55]. As in BAT, ALS patients present malformed and narrow bile ducts. The destruction and malformation of bile ducts in PBC, BAT and ALS cause chronic cholestasis and the concomitant hepatocyte death and inflammation, leading to cirrhosis or liver failure [58–60].

The prevalence of these disorders is relatively low: PBC affects 40 to 400 cases per 1,000,000 population (75-90% women), BAT affects 1 per 10,000-15,000 births in the United States and ALS 1 per 70,000 births[56,59,61]. PBC may lead to liver failure, especially after cirrhosis development and rarely to HCC (6%). Generally, the median survival from the time of diagnosis is 7-15 years[56]. Although survival of PBC patients has been largely improved, one third of patients still do not respond to medical treatments and progress to liver cirrhosis, requiring liver transplantation as the last resort for cure[58]. BAT is the most common reason for liver transplantation in children, with 60-80% of patients requiring a transplant. About one third of BAT patients that undergo liver transplant suffer complications after surgery in the first few years, being the survival rates 47-60% at 5 years and 15-35% at 10 years. Furthermore, in most cases after transplantation, patients who do not drain bile may develop progressive fibrosis, biliary cirrhosis and rarely, HCC[62]. ALS is also a poor prognosis disease. A survival rate of 20-years is estimated in 80% of patients that do not require liver transplantation and 60% of those that do require transplantation (survival after transplantation is even lower in ALS than BAT)[63].

PBC, BAT and ALS are common cholestatic disorders whose lack of effective treatment represents what occurs in most cholestatic liver diseases. Specific surgery is the standard procedure for most childhood cholestatic liver diseases although most patients develop complications in the next few years[64–66]. In addition, cholestatic liver diseases generally do not respond to any sort of medical therapy and evolve to more complicated liver conditions[67].

2.1.3 Hepatocellular carcinoma (HCC)

Liver cancer is an important cause of morbidity and mortality worldwide. According to Global Cancer Statistics, its incidence is around 782,000 new cases per year and results in approximately 745,000 deaths per year[68,69]. The most common benign liver tumors include hemangioma, hepatic adenoma and focal nodular hiperplasia. The main primary liver cancers are hepatoblastoma, cholangiocarcinoma (bile duct cancer), angiosarcoma and HCC. HCC is the main type of primary liver cancer and the fifth most common cancer. Indeed, HCC is the **second leading cause of cancer-related deaths** after lung cancer[69].

HCC is a poor prognosis cancer since it does not present symptoms in its early stages and in more than 50% of the cases is detected at an advanced stage of the disease[70–72]. At the time of the diagnosis, most HCC cases are multifocal and present underlying cirrhosis, which makes them unsuitable for surgical resection and liver transplantation[70–72]. The median survival following diagnosis is approximately 6 to 20 months but when the cancer is not completely removed, the disease is usually fatal[73]. Indeed, HCC is considered an extremely complex cancer with different signaling pathways converging in the same malignant transformation[74–77]. Even though treatment options have improved in the past few years, there is still no standard procedure for treating HCC and therapies remain palliative. HCC etiology is diverse and multifactorial and almost one-third of the cases are of unknown etiology. Chronic hepatitis B or C, alcoholism, aflatoxins and **NAFLD**[34,68,69] are some of the main risk factors for HCC development.

The major causes of DILI, NAFLD and cholestasis and the most common complications for each disease are shown in Figure 2.1. Many factors have been linked to the development of DILI, NAFLD, cholestatic liver diseases and HCC including mitochondrial metabolism alterations, which have been reported to play an essential role in the molecular bases of liver disease[17]. To this respect, a concise analysis about the involvement of mitochondria in the development of liver pathology will be provided in this next section.

2.2 MITOCHONDRIA AND LIVER DISEASE

Rolf Luft described the first mitochondrial dysfunction in 1962 in a woman with hypermetabolism[78]. Since then, the role of mitochondria in health and disease has been broadly studied. In 1963 mitochondrial deoxyribonucleic acid (mtDNA) was discovered[79] and in 1988 deletions and point mutations of mtDNA were described as causes for human diseases[80]. In addition, mutations in nuclear DNA-encoded genes involved in the correct function of mitochondria also represent major causes of a variety of pathologies[81]. The pathogenic mutation of mitochondrial genes has been associated to neurodegenerative diseases, metabolic and cardiovascular disorders, obesity, aging and cancer[82–84].

Due to the tremendous metabolic activity of the liver, **hepatocytes are one of the cell types with the highest density of mitochondria**, which make it very susceptible to disorders that affect mitochondrial functions[85,86]. Mitochondrial hepatopathies are classified depending on the cause of the liver disorder. In the primary disorders the mitochondrial defect is the primary cause of the disorder. Defects in the respiratory chain are the most frequent mitochondrial primary disorders (1 in 5,000 births) and present as ALF in the first weeks of life. In the secondary disorders, the mitochondrial damage is caused by genetic defects of non-mitochondrial proteins or by exogenous insults such as viruses, alcohol, toxins, drugs or other factors[87,88].

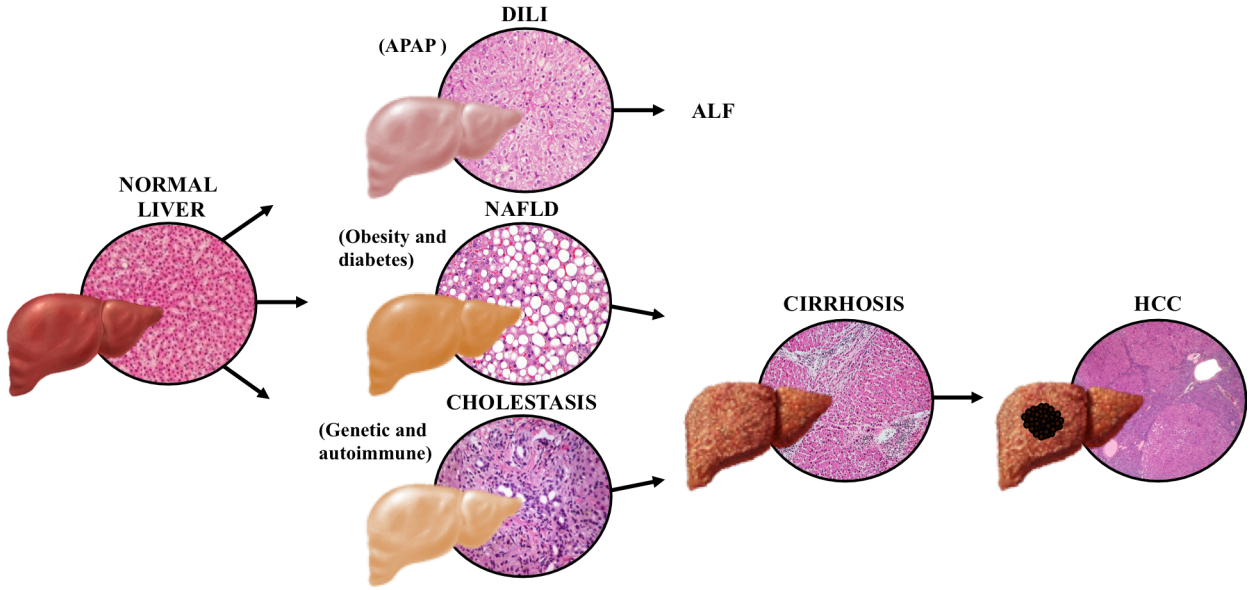

Figure 2.1 Progression of DILI, NAFLD and cholestasis to ALF, cirrhosis and HCC. In the last few years, APAP overdose, obesity and diabetes have become major causes of liver disease and consequently, the incidence of DILI and NAFLD has increased dramatically. Cholestatic liver diseases are still an important cause of liver disease and are primarily caused by genetic and autoimmune alterations.

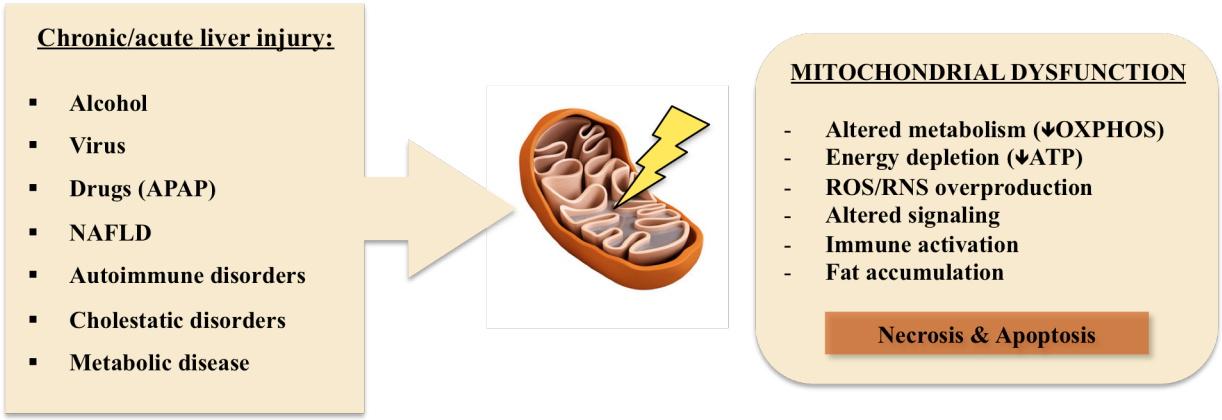

Figure 2.2 Major causes of hepatic mitochondrial dysfunction and its detrimental effects in the initiation and progression of liver disease. APAP overdose, hepatic lipid accumulation and cholestasis are important causes of mitochondrial injury. Hepatic mitochondrial damage entails cellular energy depletion, overproduction of reactive species, inflammation, steatosis and hepatocyte death, key events in the pathogenesis of liver diseases.

Indeed, mitochondrial dysfunction has been shown to play a major role in many experimental models of liver injury. For example, the transgenic expression of the hepatitis C virus core protein in mice and cell lines inhibited mitochondrial respiration and caused oxidative stress[89]. In the same way, mouse models of alcoholic liver disease (ALD)[86] and APAP hepatotoxicity[90] showed evidences of mitochondrial damage and dysfunction.

Importantly, mitochondrial dysfunction has been described as a common mechanism in the pathogenesis of several acute and chronic human liver diseases, including DILI[90], NAFLD[91], cholestatic liver diseases[92] and HCC[93]. Concretely, several studies considered these diseases the result of reactive oxygen species (ROS) overproduction due to mitochondrial dysfunction[86]. Increased ROS induce the expression of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα) and Fas ligand, and activate death-signaling pathways, like mitochondrial permeability transition and apoptosis, in the initiation and progression of the disease[6,94]. On the other hand, defects in the mitochondrial respiratory chain are known to trigger lipid accumulation within the hepatocytes and adenosine-5'-triphosphate (ATP) depletion with concomitant necrotic cell death in NAFLD, PBC and DILI[6,94]. In addition, the energetic misbalance and ROS production due to mitochondrial dysfunction largely contribute to liver fibrogenesis[95] and carcinogenesis[93]. The major causes of hepatic mitochondrial dysfunction and the harmful effects that it has on hepatocytes are shown in Figure 2.2.

All these findings highlight the major role of mitochondrial dysfunction in liver pathology. Thus, a deep study of mitochondria and its biological functions in the liver will be done.

2.3 MAIN MITOCHONDRIAL FUNCTIONS IN THE LIVER

Since they were first described by Richard Altmann in 1890 and named by Carl Benda in 1898, the importance of mitochondria mediating several fundamental cellular processes is constantly growing. Mitochondria, also called the "powerhouse" of the cell, are in charge of cellular respiration and ATP generation by oxidative breakdown of carbohydrates and FAs[96]. In addition, they regulate a multitude of different metabolic and signaling pathways in the cell, including cellular proliferation and programmed cell death[2,86].

These double membrane-bound organelles are unique as they contain their own genetic information: mtDNA, a double-stranded circular molecule of 16.5kb encoding 13 proteins (all components of the respiratory chain or the ATP synthase), 22 transfer RNAs (ribonucleic acids), and 2 ribosomal RNAs in mammals[85,87]. Furthermore, the density of mitochondria is different among the tissues depending mostly on the demand of oxidative phosphorylation (OXPHOS). For this reason, the liver, which plays a central role in most of the body's metabolic processes, is one of the richest organs in number of mitochondria. This fact makes the liver a very susceptible organ to disorders that affect mitochondrial functions[85,86].

To better understand the major role that mitochondria play in hepatic physiology and pathology, the main mitochondrial functions in the liver will be overviewed in this section.

2.3.1 Mitochondria are the main source of energy in hepatocytes

As in most eukaryotic cells, mitochondria are the main source of energy in hepatocytes. These organelles are in charge of generating **ATP**, the most abundant form of energy in the cell and considered the primary energy currency for metabolism (it is used for mostly biological processes)[96].

Carbohydrates, fats and proteins, basic components of food, are the most important energy supply molecules in mammals[2,86,97]. Mainly carbohydrates (glucose) but also lipids (FAs) and

proteins (amino acids) are converted to **acetyl-CoA**, molecule that fuels the tricarboxilic (TCA) (or Krebs) cycle to generate large amounts of energy as form of ATP and reduction power: nicotinamide adenine dinucleotide (**NADH**) and flavin adenine dinucleotide (**$FADH_2$**). The high-energy reducing molecules NADH and $FADH_2$ are further oxidized through OXPHOS by the electron transport chain (ETC) to produce more ATP[98].

The major function of carbohydrates is providing energy. Glucose enters the cell through glucose transporters (GLUT) and is initially metabolized to pyruvate via **glycolysis** (10-step reactions)[2,97]. Then, pyruvate can enter mitochondria and be converted by pyruvate dehydrogenase (PDH) to acetyl-CoA being further oxidized in the TCA cycle, or either be transformed into lactate in the cytosol by lactate dehydrogenase (LDH) when oxygen is limited, by a process known as **anaerobic glycolysis**. It should be noted that glycolysis is a major producer of ATP even if its intermediates do not enter the TCA cycle. Glucose can also be metabolized by the pentose phosphate pathway to produce the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), which serves to maintain cytoplasmic integrity and redox state through the reduction of GSH[2,99].

Providing energy is one of the important functions of lipids as well. These molecules enter the cell through different transporters like CD36 (cluster of differentiation 36) and FATPs (FA transport proteins), are then converted to acetyl-CoA in mitochondria by β-oxidation and are completely oxidized by the TCA cycle and the ETC[2]. Notably, FA metabolism leads to glycerol generation, which can be stored as glycogen.

The main steps of carbohydrates and lipids metabolism are summarized in Figure 2.3.

2.3.1.1 The TCA cycle

The TCA cycle is the **central pathway of oxidative metabolism** and an important source of energy, intermediates for biosynthesis and reducing equivalents to the respiratory chain in all aerobic organisms. It is thus considered at the same time an anabolic and catabolic pathway. In eukaryotic cells it takes place in mitochondria. The TCA cycle is the second step of the catabolism of carbohydrates, lipids and proteins[2,97,100]. As already mentioned, before entering the cycle, these macromolecules are converted into acetyl-CoA. Then, acetyl-CoA is oxidized up to CO_2 through a cascade of enzymatic oxidative reactions (the TCA cycle) that lead to energy release, mainly as form of ATP and the high-energy reducing molecules NADH and

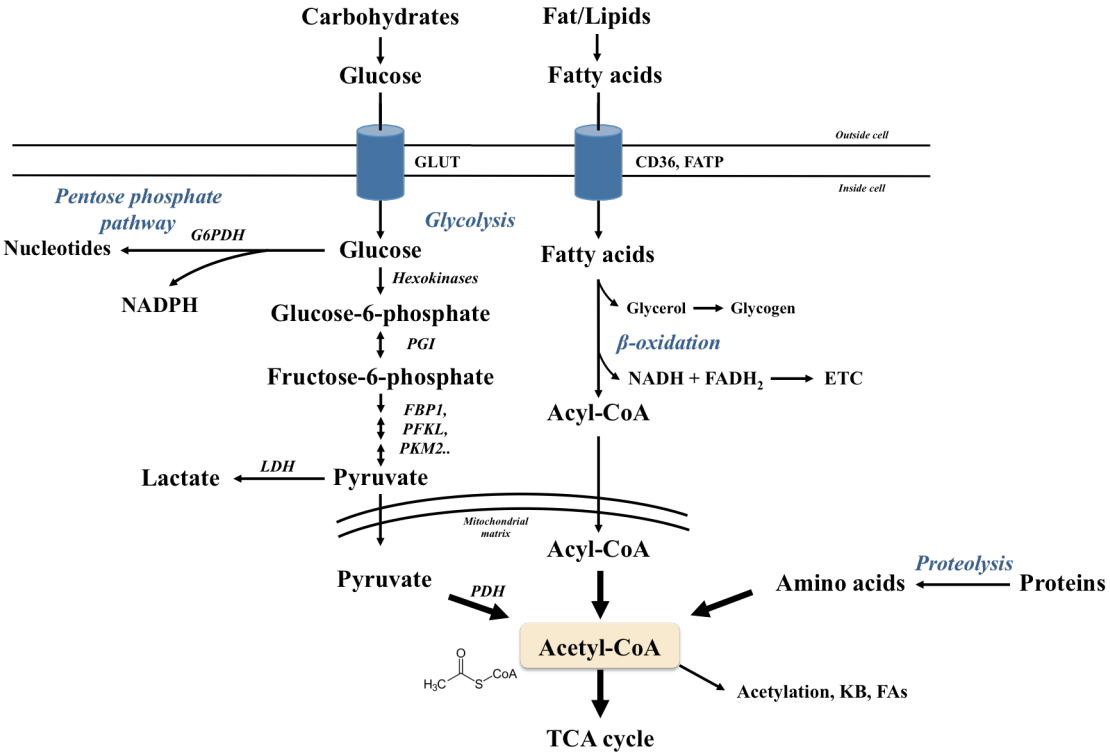

Figure 2.3 Carbohydrates, lipids and proteins are an important source of acetyl-CoA. Glucose, which is the most important energy supply molecule in mammals, is metabolized by glycolysis or the pentose phosphate pathway in the cytosol to produce pyruvate, nucleotides and NADPH. Following glycolysis, pyruvate can enter mitochondria being further oxidized as acetyl-CoA in the TCA cycle, or undergo anaerobic glycolysis and form lactate. Lipids, which represent another major source of energy, are metabolized by β-oxidation leading to acetyl-CoA along with NADH and $FADH_2$, reducing molecules that fuel the ETC. During β-oxidation glycerol is produced and can be stored as glycogen. Proteins are also metabolized by different pathways to produce acetyl-CoA. Although most acetyl-CoA is oxidized in the TCA cycle to produce ATP, it can also be used for ketogenesis, acetylation and FA synthesis *de novo*.

$FADH_2$. The third and last step is OXPHOS, where the reducing power (NADH and $FADH_2$) is used for the synthesis of ATP[98]. Remarkably, the TCA cycle also has a central role in gluconeogenesis, lipogenesis, and interconversion of amino acids[100].

The TCA cycle consists on 8 major steps catalyzed by 8 enzymes (Figure 2.4):

Step 1: acetyl-CoA joins oxalacetate to form citrate and a molecule of water leads to the release the CoA group and the formation of citrate. Citrate synthase catalyzes this process. **Step 2:** citrate is converted to isocitrate by aconitase. **Step 3:** isocitrate is oxidized to α-ketoglutarate by the isocitrate dehydrogenase (IDH), an important regulatory enzyme of the TCA cycle. **Step 4:** α-ketoglutarate is oxidized and a CoA group is added to form succinyl-CoA. The enzyme catalyzing this step is α-ketoglutarate dehydrogenase (α-KGDH), another important enzyme in the regulation of the TCA cycle. **Step 5**: the CoA group is removed from succinyl-CoA to produce succinate by the succinyl-CoA synthethase. **Step 6:** succinate is oxidized to fumarate by the succinate dehydrogenase (SDH). SDH is the complex II of the ETC and directly transfers the $FADH_2$ electrons into the chain. **Step 7:** fumarase catalyzes the addition of a water molecule to fumarate to produce L-malate. **Step 8:** oxalacetate is regenerated by oxidation of L-malate by malate dehydrogenase (MDH).

Each molecule of acetyl-CoA that enters the TCA cycle leads to the production of three molecules of NADH, one of $FADH_2$ and one molecule of ATP. In addition, the oxidation of each molecule of NADH and $FADH_2$ by the ETC produces respectively 3 and 2 molecules of ATP. Thus, the final energy balance is 12 molecules of ATP per molecule of acetyl-CoA. For this reason, the TCA cycle is considered the most important and efficient metabolic pathway for energy supply[101].

The TCA cycle is essential for many biological processes and major metabolic pathways converge on it[102]. Thus, it must be tightly regulated to avoid the overproduction of large amounts of energy. The availability of substrates and the inhibition of products are the main regulators of the TCA cycle[103]. Indeed, high concentrations of ATP and the reducing molecules NADH and $FADH_2$ inhibit the production of acetyl-CoA. Moreover, these molecules can remain bound to the enzymes that produce them in order to block the degradation of substrates if the energy demand is not high enough. NADH, product of all dehydrogenases with the exception of SDH, inhibits PDH, IDH, α-KGDH, and also citrate synthase. In the same way, acetyl-CoA inhibits PDH, while succinyl-CoA inhibits α-KGDH and citrate synthase. Finally, the citrate synthase, enzyme that catalyzes the first step of the cycle only in one way, requires the presence of oxalacetate and acetyl-CoA to initiate the pathway.

Because of its strategic situation in the body, the liver is the first organ that receives the nutrients absorbed in the intestinal tract and the responsible for the catabolism and distribution of glucose, FAs and amino acids[3,97]. For this reason, most metabolic pathways take place in the liver, where hepatic TCA cycle is considered central[103]. A unique feature of hepatic mitochondria is that the TCA cycle is not always coupled to β-oxidation: acetyl-CoA in excess can be shunted to ketogenesis[2]. Hence, the TCA cycle continues coupled to cellular respiratory demand while ketogenesis dissipates excess acetyl-CoA into ketone bodies (KB), which are additional metabolic fuels for extrahepatic tissues during fasting.

Importantly, impaired TCA cycle function, which can be caused indirectly by mitochondrial dysfunction or directly by disturbances in components of the cycle like decreased activities of specific enzymes,

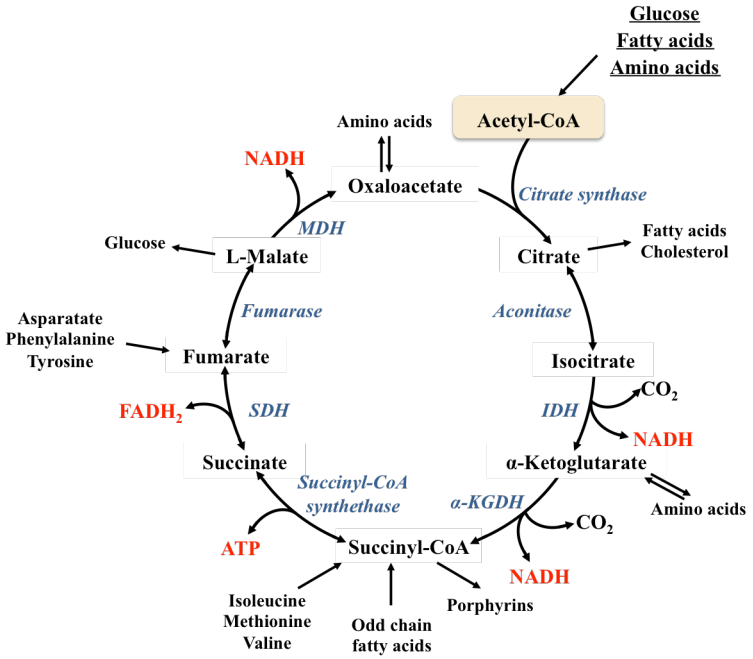

Figure 2.4 The TCA cycle. The TCA or Krebs cycle is the central pathway of oxidative metabolism and an important source of energy, intermediates for biosynthesis and reducing equivalents to the respiratory chain in all aerobic organisms. Initially and before entering the cycle, carbohydrates, lipids and proteins are broken down and converted into acetyl-CoA through different pathways. Then, acetyl-CoA is oxidized up to CO_2 through an 8 step-cascade of enzymatic oxidative reactions in mitochondria, leading to energy release, as form of ATP and the high-energy reducing molecules NADH and $FADH_2$. For each molecule of acetyl-CoA that enters the TCA cycle, 3 molecules of NADH, one molecule of $FADH_2$ and one molecule of ATP are produced. All the steps, enzymes, intermediates and products of the TCA cycle are shown.

has been reported in a variety of liver diseases including DILI[104], NAFLD[105] and HCC[106,107]

2.3.1.2 The electron transport chain

The ETC is a complex machinery where the final step of aerobic respiration takes place[108,109]. The process of OXPHOS, in which NADH and $FADH_2$ oxidation leads to ATP production, is carried out by the components of the ETC: the complexes I, II, III, IV and the ATP synthase (complex V). All these complexes are formed by multiple subunits and are located in the mitochondrial inner membrane. During OXPHOS, electrons (e^-) and protons (H^+) are transferred simultaneously as products of redox reactions, creating an electrochemical proton gradient that drives the synthesis of ATP. All the processes and complexes are shown in Figure 2.5.

The **complex I** (NADH-ubiquinone oxidoreductase) is the enzyme responsible for the oxidation of NADH. NADH is oxidized to NAD^+ by reducing Flavin mononucleotide (FMN) to $FMNH_2$ and electrons are transferred via iron-sulfur (Fe-S) clusters to ubiquinone (coenzyme Q or CoQ). At the same time, four H^+ are translocated from the matrix to the intermembrane space.

The **complex II** (succinate dehydrogenase or SDH) is an important enzyme in mitochondrial metabolism since it is involved both in the ETC and the TCA cycle. This enzyme provides an additional entry point of e^- into the ETC by the oxidation of $FADH_2$ and catalyzes the oxidation of succinate in the TCA cycle, leading to $FADH_2$ production. Upon $FADH_2$ oxidation, $2e^-$ are transferred to Coenzyme Q (CoQ). Unlike complex I, no H^+ are pumped to the intermembrane space in this reaction. Even though the complex I has always been considered the main energy contributor to the overall ETC process, complex II has recently been detected as the most important complex in the ETC process in the liver[110].

The **Complex III** (ubiquinone-cytochrome-c oxidoreductase) catalyzes the reduction of Cyt *c* by ubiquinol oxidation to ubiquinone with the concomitant translocation of four H^+ into the intermembrane space and then, the **complex IV** (cytochrome *c* oxidase) accomplishes the last transfer of e^- from Cyt *c* to O_2 producing water and the pump of two H^+ across the membrane.

Finally, the **complex V** (ATP synthase) makes use of the proton gradient created by the ETC and transports down a H^+ to complete the phosphorylation of ADP to ATP.

a) Role of proton leak

It is known that mitochondria couple respiration to ATP production by the transmembrane electrochemical proton gradient created by the pump of H^+ of complexes I, III and IV into the intermembrane space during OXPHOS. However, since H^+ can return to the matrix independently of ATP synthase, **OXPHOS and ATP synthesis are not completely coupled**[111,112] (Figure 2.5). Interestingly, this incomplete coupled OXPHOS represents another mechanism to maintain energetic metabolism homeostasis and body function.

The basal proton leak, which is unregulated, is specific to cell type and correlates with its metabolic rate. However, proton leak can also be inducible by the adenine nucleotide translocase (**ANT**) and the uncoupling proteins (**UCPs**), and activated by superoxide, peroxidation products or FAs[111]. The coupling efficiency of hepatocytes is around 80%, which means that basal proton leak accounts for 20% of the resting metabolic rate of hepatocytessimilar to other cell types[111]. Although the physiological role of proton leak has not been completely described, it has been showed that it exists to minimize oxidative damage by mitigating mitochondrial superoxide production. Besides this, proton leak has been associated to a variety of processes

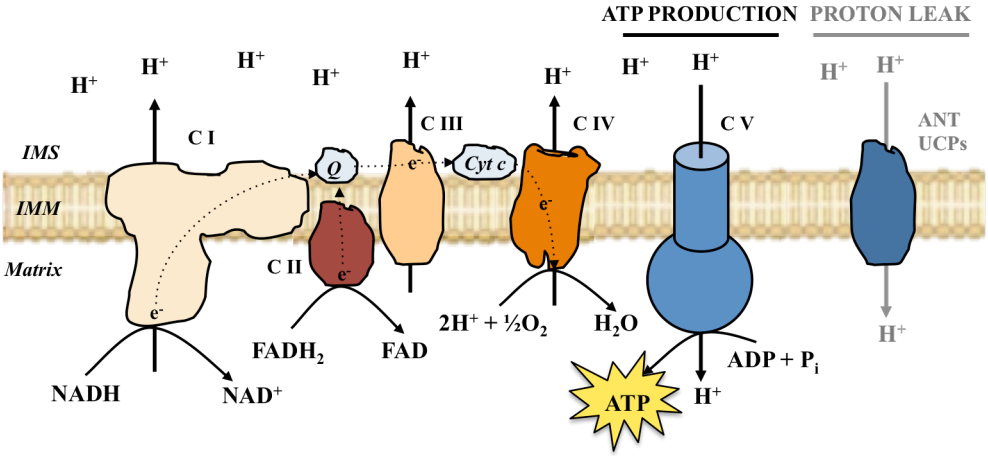

Figure 2.5 ETC and proton leak. The ETC is a series of electron transporters (complexes I-V) embedded in the IMM that shuttle electrons from NADH and $FADH_2$ to molecular oxygen. In the process, protons are pumped from the mitochondrial matrix to the intermembrane space, and oxygen is reduced to form water. ATP synthase (complex V) transports down a H^+ to complete the phosphorylation of ADP to ATP. Protons can also return to the matrix independently of ATP synthase through uncoupling proteins, process known as proton leak.

including thermogenesis in brown adipose tissue, where the leak of H^+ is induced by UCP1[113]. Nowadays, proton leak is being used as a therapeutic target for a variety of diseases including obesity, type 2 diabetes and other diseases related to metabolic imbalance and oxidative stress[113].

b) Supercomplex formation

In 1955, Britton Chance and G. R. Williams proposed for the first time that respiratory enzymes could aggregate into larger complexes[114]. But it was in 2000, when Hermann Schägger detected using blue native-polyacrylamide gel electrophoresis (BN-PAGE) techniques in bovine mitochondrial membrane proteins, that ETC complexes can assembly and form supramolecular structures, called supercomplexes (SC)[115]. Complexes I, III and IV (but not complex II although its presence has recently been suggested[116,117]), which are considered free-moving entities, can aggregate in different ways to form SC or remain in a free state as shown in Figure 2.6[118,119]. The most common SC are complexes I/III/IV (respirasome), complexes I/III and complexes III/IV. Complex V can be isolated as a dimer but rarely as part of the SC. Like in the fluid model, CoQ and Cyt *c* act as mobile carriers that move along the membrane.

Although the exact role of SC is unclear, it is well known that their formation is dynamic and that their organization **optimize the use of subtrates**, making the electron flux more effective and reducing the leakage of e^- that lead to the formation of ROS. Thus, metabolic organs like the heart and the liver are rich in SC. Interestingly, increased formation of SCs has been observed in response to energy demanding conditions, such as fasting[120].

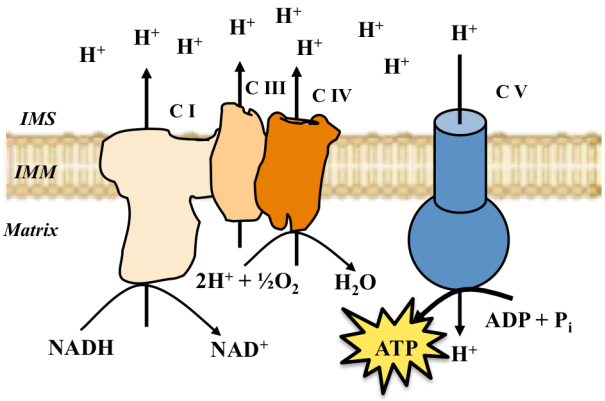

Figure 2.6 Respiratory supercomplexes. Complexes I, III and IV assembly into supercomplexes (SC) or respirasomes facilitating the transport of e^- and reducing the production of ROS.

2.3.1.3 Fatty acid β-oxidation and ketogenesis

As already commented, lipids are an important source of energy in the body[2,97]. These organic compounds are catabolized mainly by β-oxidation, an intramitochondrial metabolic pathway that produces huge amounts of energy (a molecule of palmitate generates around 129 ATP equivalents upon oxidation), and can be synthesized by *de novo* lipogenesis in the liver and stored as reserve of energy[2]. When entering the body, dietary lipids, which are mainly composed by triglycerides (TGs), are digested and absorbed as monoglycerides and FAs. At this point and depending on the metabolic state, FAs can be converted to TGs and stored as fat in the adipose tissue or be rapidly metabolized. During fasting, these adipose tissue stored TGs are released and metabolized in the liver to be used as energy source. In the liver, FAs must be activated by FA acyl-CoA synthetase (FACS) to acyl-CoA in the cytosol to be translocated into mitochondrial matrix where they undergo β-oxidation: acyl-CoA is converted to acetyl-CoA, which can enter the TCA cycle and produce energy as ATP. Short- and medium-chain FAs can enter the mitochondrial matrix without being activated. However, long-chain FAs must be translocated across the mitochondrial membrane through the carnitine palmitoyltransferase-1 (**CPT1**). Thus, generally, short-, medium and long-chain FAs are β-oxidized in mitochondria (Figure 2.7). On the contrary, very-long-chain FAs are oxidized within peroxisomes. And alternatively, medium-chain FAs undergo instead of β-oxidation, ω-oxidation in the endoplasmic reticulum (ER)[121].

Since β-oxidation is an energy-producing process, it is expectable that it can be activated under energy-demanding conditions[122]. Peroxisome proliferator-activated receptor alpha (**PPARα**) is a major transcriptor factor regulating lipid metabolism in the liver by inducing the expression of a battery of genes that participate in the process. It is activated under conditions of energy deprivation and promotes the uptake, utilization and β-oxidation of FAs by upregulating genes involved in FA transport like CPT1 (carnitine palmitoyltransferase 1A), FA binding and activation like ACADM and ACADL (acyl-CoA dehydrogenase, medium and long chain) and peroxisomal and mitochondrial FA β-oxidation[123].

In cases of increased FAs uptake, the liver can produce large amounts of KB (acetoacetate, β-hydroxybutyrate and acetone) by ketogenesis. During this process, excess acetyl-CoA is converted to KB instead of being oxidized in the TCA cycle, which represents an important mechanism to supply energy to certain organs (particularly the brain). When demanded KB can be converted into acetyl-CoA by ketolysis[2] (Figure 2.7).

The liver plays a major role in the regulation of fat metabolism importing FAs and processing, storing and exporting lipids[2]. For this reason, it is very susceptible to disturbances in lipid homeostasis, which represent a major and common feature in hepatic diseases[124]. β-oxidation is directly linked to ETC. Thus, mitochondrial dysfunction, which is implicated in the pathogenesis of most liver diseases, impairs the oxidation

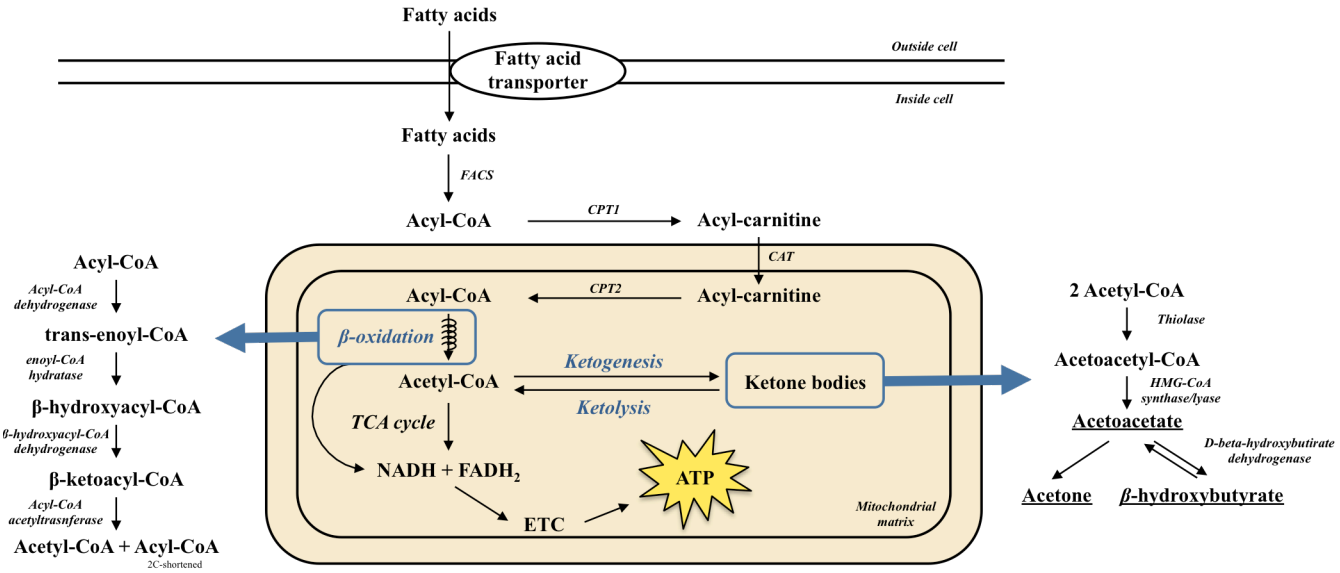

Figure 2.7 β-oxidation and ketogenesis. FAs are transformed into acyl-CoA in the cytosol and translocated into mitochondrial matrix to undergo β-oxidation. Acyl-CoA is converted by a series of oxidation reactions to acetyl-CoA. Then, acetyl-CoA can enter the TCA cycle and produce energy or be converted into KB by ketogenesis to store energy. Under energy demanding conditions, KB can be converted into acetyl-CoA by ketolysis. Short- and medium-chain FAs can enter the mitochondrial matrix without being activated, however long-chain FAs must be translocated across the mitochondrial membrane through CPT1.

of FAs leading to lipid accumulation within the liver (steatosis). Defective β-oxidation has been described in DILI[125], advanced NAFLD[91] and cholestatic disorders[126].

2.3.2 Mitochondria control ROS homeostasis

ROS are highly reactive chemical radicals generated as products of the normal metabolism of oxygen. There are many different species such as peroxides (H_2O_2), superoxide ($\bullet O_2^-$), hydroxyl radical (•OH) and single oxygen that play important roles in cell signaling and homeostasis[127]. Under stress circumstances like alcohol and drug (APAP) toxicity, hydrophobic BAs accumulation, ultraviolet radiation, viral infections etc, ROS can reach dramatic levels that may damage cellular proteins, lipids and DNA, becoming cytotoxic[128]. If there is too much damage, the cell may undergo apoptosis or even necrosis[6]. On this basis, damage to DNA by ROS has been widely accepted as a major cause of cancer[83]. Mitochondria are the main source of ROS in most mammalian cells. Indeed, ROS produced by mitochondria are known as mitochondrial ROS (**mROS**)[129]. Besides ROS, reactive nitrogen species (RNS) represent another type of highly reactive radicals that can also lead to cellular damage. RNS derive from the reaction of superoxide with nitric oxide (NO), which produces peroxynitrite, a powerful oxidant[130].

"Oxidative stress" is known as the imbalance between the production of reactive species and their detoxification by the antioxidant system, and has been associated with the development of many diseases and most hepatopathies[128]. Importantly, these radicals (especially those derived from oxygen) can also be beneficial for the organism. They can act as messengers in signal transduction pathways and regulate gene expression, the immune response, inflammation, cell proliferation and apoptosis[131,132].

2.3.2.1 Production of ROS

ROS can be exogenous (those produced by radiation, drugs and activated macrophages and neutrophils for example) and endogenous. The latter, are produced intracellularly by multiple mechanisms. One of the main generators of ROS in the cell are NADPH oxidases (**NOX**), which are localized in cell membranes, mitochondria, peroxisomes and ER[133]. Cytochromes P450 (**CYPs**), which are found mainly in ER membranes within hepatocytes, are also involved in ROS production[134]. These enzymes catalyze reactions where organic substrates are oxidized while oxygen is reduced to water.

Other major producer of ROS (more concretely, the major source of mROS) is the ETC, where the processes of OXPHOS and ATP synthesis take place[109,135]. OXPHOS, which has been previously described, consists on different transfers of e^- and H^+ (products of redox reactions) through the components of the ETC, where each acceptor has a higher reduction potential than the previous. Although in normal conditions the last e^- passing through the chain reduces the O_2 to produce water, around 1-2% of e^- leak from the chain and incompletely reduces O_2, leading to the formation of the superoxide radical. This has been documented mostly in complexes I and III and also in complex II[109,135,136]. Although superoxide is not a particularly reactive radical, it is the precursor of most other ROS and can activate oxidative chain reactions.

2.3.2.2 Antioxidant system

The liver, an organ rich in mitochondria due to its high metabolic activity, is thereby particularly susceptible to damage by ROS[86]. In order to be protected against the cytotoxicity of reactive radicals, organisms have evolved and developed an antioxidant defense system over the time[137]. Thus, mammalian cells possess a variety of antioxidant enzymes and molecules that can metabolize these radicals[131].

Some of the most important antioxidant enzymes are: superoxide dismutases (**SOD**), a family of metalloproteins that convert superoxide to H_2O_2; **catalase** and glutathione peroxidases (**GPx**), which catalyze the reaction from H_2O_2 to water; glutathione reductase (**GRd**), that transforms oxidized glutathione (GSSG) to its reduced form GSH, and glucose-6-phosphate dehydrogenase (**G6PDH**), responsible of the formation of NADPH through the pentose phosphate pathway[138]. Moreover, these enzymes cooperate in detoxifying ROS.

GSH is the main antioxidant in the cell and is able to prevent cellular damage by ROS neutralization, donating a reducing equivalent from its thiol group of cysteine to unstable species[139]. Glutathione exists in both reduced (GSH) and oxidized (GSSG) states. Importantly, once oxidized, GSSG can be reduced again to GSH using NADPH as electron donor to maintain the pool of GSH (in healthy cells, 90% of total glutathione is GSH). Indeed, the ratio GSH/GSSG is used as an indicator of oxidative stress[140]. Besides ROS neutralization, GSH can conjugate to toxic compounds and metabolites for detoxification, like for example the reactive metabolite of APAP: NAPQI in the liver. Since intracellular GSH levels can be easily depleted due to increased ROS or reactive metabolites, the restoration of its levels (using *N*-acetylcysteine or NAC) is a therapy commonly used for the treatment of many diseases[141]. Another important defense against oxidative stress is the activation of the NF-E2-related factor 2-antioxidant response element (**NRF2-ARE**) signaling pathway, which regulates the expression of genes involved in detoxification and elimination of ROS[142].

Most chronic liver diseases are characterized by increased ROS, regardless of their etiologies[86]. This is because the liver is particularly susceptible to damage by ROS. Moreover, all hepatic cell types are involved in oxidative damage: high ROS levels can induce hepatocyte and endothelial cells apoptosis and hepatic stellate cell (HSC) and Kupffer cell (KC) activation, contributing to the pathogenesis of inflammatory, metabolic and proliferative liver diseases[131]. Oxidative damage is considered an underlying cause of DILI[143], NAFLD[35], cholestatic disorders[144] and HCC[145].

Multiple studies have shown patterns of expression of proteins in response to oxidative stress. In the liver, the activation of the mitogen-activated protein kinase (MAPK) family of proteins, particularly p38 and c-Jun N-terminal kinase (**JNK**), seems to play a pivotal role in the activation of redox transcription factors like nuclear factor-kappaB (NFκB) and activator protein-1 (AP-1) and the subsequent chain reactions that result in hepatocyte apoptosis[127,146,147]. On the other hand, studies in animal models have demonstrated the important role of NRF2-ARE signaling pathway in counteracting the development of many liver diseases including DILI, NAFLD, cholestasis, fibrosis and cancer[145]. Moreover, NRF2 supports liver regeneration[148].

Undoubtedly, ROS-related injury is crucial in the pathogenesis of liver diseases[131,132].

2.3.3 Mitochondria regulate calcium homeostasis

Calcium (Ca^{2+}) is the most abundant mineral in human body and is essential for the normal physiology of the organism. It is involved in multiple signal transduction pathways where it acts as second messenger, specifically for nerve signal transmission and muscle contraction. It is also required from many enzymes as a cofactor. The main site of storage of calcium in the body is the bone (99%), from where it can be released into bloodstream when needed. The other 1% remains in the blood where its levels must be tightly regulated in order to maintain vital physiological functions[149].

Increased levels of intracellular calcium regulate many liver functions. Interestingly, depending on its subcellular localization calcium can control different processes, like bile secretion by cytosolic Ca^{2+}, glucose and energy metabolism by mitochondrial Ca^{2+} and cell cycle, proliferation and apoptosis by nucleopasmic Ca^{2+}. Besides controlling metabolism, respiration and ROS, mitochondria contribute to the tight regulation of calcium signals by maintaining cytosolic calcium levels[150]. Importantly, mitochondrial calcium might affect mitochondrial membrane potential (MMP), ROS production, the activity of dehydrogenases that participate in the TCA cycle like IDH and α-KGDH and thus globally, mitochondrial metabolism[151]. Recent studies have shown how defects in calcium signaling play a role in the pathogenesis of a wide range of liver diseases. Two examples of this are HCC where mitochondrial calcium signals regulate hepatocyte proliferation but can impair cell growth, and cholestasis, where reduced expression of some canalicular transporters is due in part to the loss of calcium signaling[150].

2.3.4 Regulation of cell viability and death

As commeted before, hepatocytes are especially susceptible to injury due to its central role in clearance and metabolism. Indeed, hepatocyte death is an important event associated with the initiation and development of most liver diseases[6]. It should be noted that cell death can also occur as a mechanism of defense to eliminate damaged cells[152]. It is widely known that mitochondria are essential in regulating the balance between cell survival and cell death in hepatocytes. In fact, they

control the two major processes leading to hepatocyte cell death: **apoptosis** and **necrosis**[6,94].

2.3.4.1 Apoptosis: programmed cell death

Apoptosis is the programmed and energy-dependent process by which cells suffer morphological changes like membrane blebbing, shrinkage, chromatin condensation and DNA fragmentation that lead to cell death. In the liver, drugs, alcohol, toxic BAs and lipids are good examples of factors that may induce hepatocyte apoptosis[153]. Depending on the etiology, hepatic apoptosis can initiate in a different manner and its pathophysiological role vary during liver injury. Enhanced and chronic hepatic apoptosis is associated with inflammation and fibrosis and can also predispose to cancer development[154]. As just mentioned, apoptosis can initiate by different pathways: the **intrinsic** (mitochondrial) or **extrinsic** (death receptors) pathway. In the last years, evidence of a crosstalk between the two pathways has been described[155].

a) Intrinsic or mitochondrial pathway

The intrinsic apoptotic pathway, also known as the mitochondrial apoptotic pathway, can be initiated by different stimuli like radiation, hypoxia, toxins, viral infections and reactive radicals. As shown in Figure 2.8, the intracellular signals produced by these stimuli cause the opening of the mitochondrial membrane transition pore (MPTP), which leads to the loss of MMP and the release of the pro-apoptotic mitochondrial proteins, cytochrome *c* (Cyt *c*) and Smac/DIABLO, into the cytosol. Cyt *c* drives the formation of the caspase-activating complex called "**apoptosome**" by apoptotic-protein activation factor-1 (Apaf-1) and procaspase-9 binding and activation. Once activated, this initiator caspase can then activate effector caspases, like **caspase-3**, and trigger a cascade of events leading to apoptosis. On the other hand, Smac/DIABLO induce apoptosis by blocking the activity of the inhibitors of apoptosis proteins (IAPs)[6,154].

There is a second group of proteins that are released from the mitochondrial matrix into the cytosol during apoptosis: apoptosis inducing factor (AIF), endonuclease G and caspase-activated DNase (CAD). Unlike Cyt *c* and Smac/DIABLO, the release of these proteins is a late event that occurs after the cell has been programmed to die[156]. During apoptosis, these proteins translocate into the nucleus where they produce DNA fragmentation and chromatin condensation[6,154].

Members of the B-cell lymphoma-2 (**Bcl-2**) family of proteins[157] and tumor suppresor **p53**[158] regulate mitochondrial apoptotic events. Bcl-2 proteins control the permeability of the mitochondrial membrane (MPTP formation) and might either promote or inhibit apoptosis. Thus, they control Cyt *c* release and are considered the final determinants for cell death. Some anti-apoptotic proteins of this family are Bcl-2 and Bcl-xL and pro-apoptotic Bax, Bad, Bim and Bid. One example mechanism of action of these proteins is the heterodimerization of Bad with Bcl-xL or Bcl-2 to inhibit their anti-apoptotic activity and then induce cell death[159]. Puma (p53 upregulated modulator of apoptosis) and Noxa are also members of the Bcl-2 family. These pro-apoptotic proteins are activated by p53 and play major roles in mediating cell death triggered by genotoxic damage and oncogene activation[157,158].

ROS-induced mitochondrial apoptosis is a critical event in the pathogenesis of most liver diseases including DILI, NAFLD, cholestasis and HCC. In the liver, the mitochondrial apoptotic pathway is strictly linked to the extrinsic pathway.

b) Extrinsic pathway: Death-receptors

On the contrary to the intrinsic apoptotic pathway, this signaling pathway involves transmembrane death receptors. CD95, also known as **Fas receptor**, tumor necrosis factor receptor 1 (**TNFR1**) and death receptors 4/5 (**DR4/5**), which all belong to the TNF superfamily of receptors and are characterized by having a death domain (DD), are the most important mediators of apoptosis in the liver. Upon ligand binding, death receptors oligomerize, recruit Fas-Associated protein with Death Domain (FADD) and form the death-inducing signaling complex (DISC), which activates the procaspase-8/10, that after cleaves Bid to *t*Bid and leads to mitochondrial permeabilization and apoptosis[6,154].

Due to the ubiquitously expression of death receptors in the liver, hepatocytes are especially susceptible to death-receptor mediated apoptosis[94]. It has been shown in *in vitro* and *in vivo* experimental models of extrahepatic cholestasis, that toxic BAs can trigger hepatocyte apoptosis by death-receptor pathways independently of ligands. BAs promote Fas or DR5 oligomerization and the subsequent activation of the apoptotic pathway[160]. In the same way, in experimental models of NAFLD, it was observed that FFA upregulate Fas, TNFα, TNFR1 and DR5 expression and increase the sensitivity of hepatocytes to death receptors-mediated apoptosis[161].

c) JNK pathway

JNKs belong to the family of MAPKs and are involved in the regulation of cell proliferation, differentiation and apoptosis[162]. In the liver, the implication of JNK signaling in hepatocyte damage and death is considered critical. Indeed, JNK enzymes (JNK1 and JNK2) are associated to both the mitochondrial and the death-receptor apoptotic signaling pathway and can be activated by many factors including cytokines, death receptors and ROS[147,163]. Notably, it has been shown that JNK transitory activation is pro-survival, while its sustained activation promotes cell death[146]. Hepatic JNKs can activate pro-apoptotic signaling pathways by inducing the expression of pro-apoptotic genes through specific transcription factors and also by regulating the activity of different pro-apoptotic or pro-survival mitochondrial proteins. For instance, JNK may induce cell death by regulating proteins of the Bcl-2 family (e.g

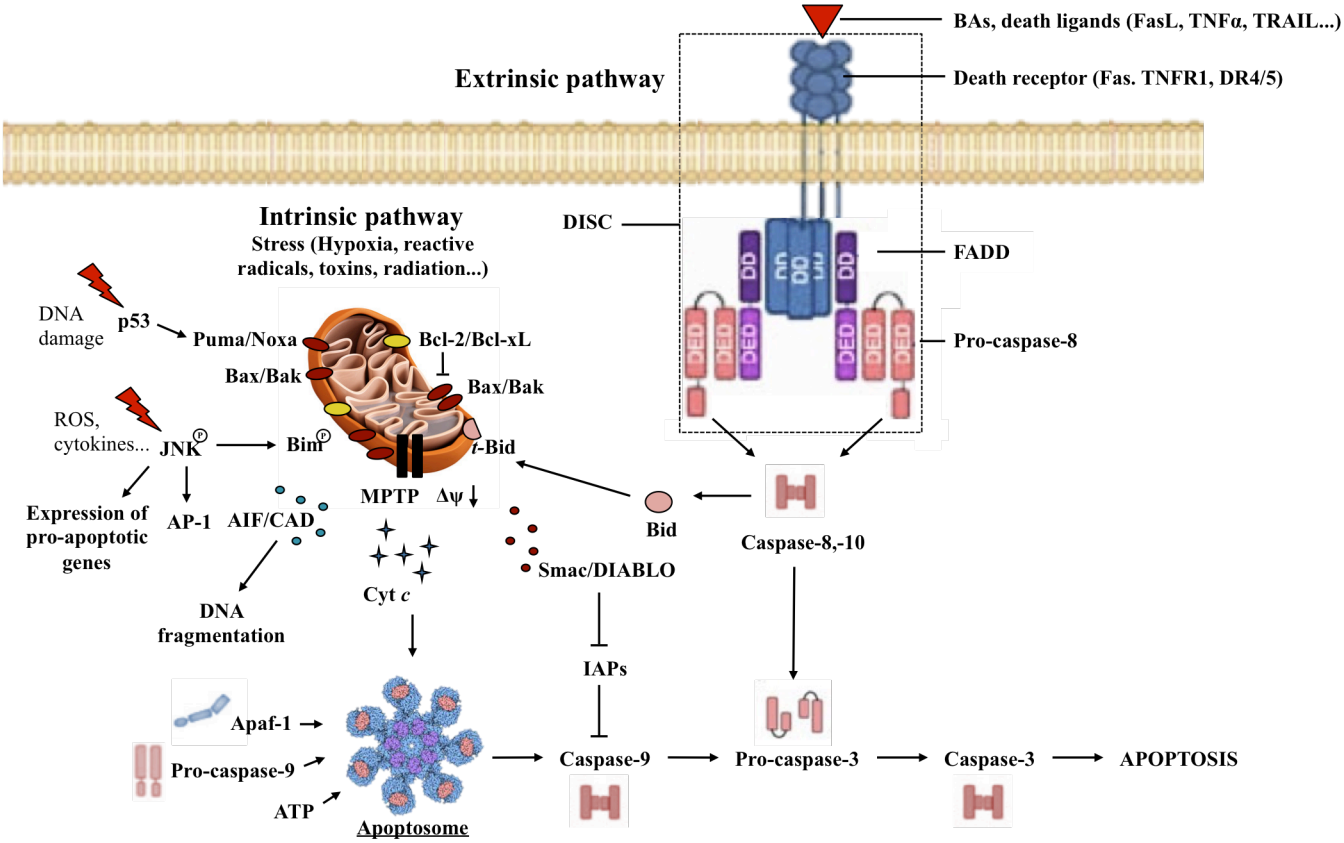

Figure 2.8 Programmed cell death. Depending on the etiology, hepatic apoptosis can be initiated by the intrinsic (mitochondrial) or the extrinsic (death receptors) pathway. The activation of JNK is associated to liver injury and strongly involved in the hepatic apoptotic process. In the liver, drugs, alcohol, BAs and lipids are important factors that can cause cell death. Upon mitochondrial stress, Bcl-2 proteins form MPTP and pro-apoptotic proteins (Cyt *c*, Smac/DIABLO and AIF/CAD) are released into the cytosol. Cyt *c* drives the formation of the apoptosome by Apaf-1 and pro-caspase-9, which activates effector caspases and triggers the caspase cascade leading to apoptosis. Smac/DIABLO induce apoptosis by inhibit IAPs and AIF/CAD, once apoptosis is initiated, translocate into the nucleus to fragment DNA. On the other hand, death ligands and toxic BAs promote death receptors oligomerization, the formation of DISC and at the same time, the activation of caspases and the opening of MPTP. Finally, JNK, which can be activated by different stimuli, regulates the expression of pro-apoptotic genes, while regulating the activity of a variety of mitochondrial pro-apoptotic and pro-survival proteins.

Bim phosphorylation), by degrading c-FLIP (inhibitor of death-receptor signaling) or by activating AP-1[162,163].

Summing up, **JNKs** are closely associated with liver injury and are significantly **implicated in the pathogenesis of many liver diseases**. Both JNK1 and JNK2 play major roles in obesity and steatosis, APAP and alcohol-induced liver injury, cholestasis and cancer development[147,163–165].

2.3.4.2 Oncotic necrosis

Necrosis is the other main pathway of cell death. Unlike apoptosis, it is not energy-dependent and is typically caused by metabolic perturbations and ATP depletion. There are not well-defined pathways mediating this process and is thus considered, an accidental way of cell death caused by disruptions of many different pathways at the same time. In the liver, ischemia-reperfusion and DILI are two examples of hepatocyte necrosis-related liver injury[6,94]. Although BAs toxicity is usually associated to apoptosis, in the experimental model of obstructive cholestasis by bile duct ligation (BDL), mice display necrotic areas in the liver indicating that necrosis also occurs in cholestasis[6].

As shown in Figure 2.19, this process, frequently referred as "oncotic necrosis", is characterized by cellular swelling, blebbing and the final rupture of the plasma membrane. This rupture is accompanied by the opening of the MPTP and disturbance of membrane electrical and ion gradients along with the release of metabolic intermediates and cytosolic enzymes into the extracellular environment (Damage-associated molecular patterns (DAMPs), Cyt *c*…), which may induce a **high inflammatory response**. Although there are not well-established signaling pathways underlying necrosis a new form of "programmed" necrosis (named necroptosis) has been described and some proteins like the receptor interacting protein (RIP) family of proteins that interact with death-receptors and caspases have been implicated in the process. Necrostatin-1 represents a potent inhibitor of RIP family that is being used in necrotic injury models[6,166].

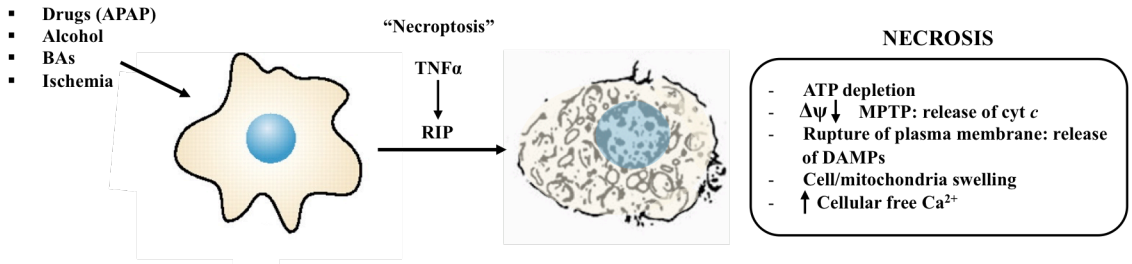

Figure 2.9 Oncotic necrosis. Necrosis is a major pathway of hepatocyte death and an important event in a variety of liver diseases. Multiple factors like APAP, toxic BAs and high levels of ROS trigger necrosis by inducing cell and mitochondria swelling, loss of OXPHOS with subsequent ATP depletion, MPTP opening and rupture of plasma membrane lead to the release of metabolic intermediates and cytosolic enzymes, among other processes. Unlike apoptosis, necrosis is considered accidental and ATP-independent and is associated to a stronger inflammatory response. In the last few years proteins like RIP have been involved in what is called "necroptosis".

In spite of having very different underlying molecular mechanisms, mitochondrial dysfunction and depolarization represent crucial events in both apoptosis and necrosis.

2.3.5 Ammonia detoxification

Ammonia (NH_3) is a toxic compound that is formed as waste byproduct of the metabolism of amino acids, mainly of the synthesis of glucose from amino acids. Since its accumulation can be lethal, it must be rapidly removed from the body. Ammonia is specifically toxic to the central nervous system because its reaction with α-ketoglutarate to produce glutamate depletes the levels of α-ketoglutarate and impairs the TCA cycle in neurons[167].

The liver is the responsible of ammonia detoxification[86]. In fact, hepatic mitochondria are the only that contain enzymes that allow the conversion of ammonia to urea and its elimination from the circulation. Hence, in advanced liver disease and acute liver failure, conditions where liver function is disrupted, increased levels of ammonia in blood can lead to hepatic encephalopathy (HE). Importantly, HE appears in one third of cirrhotic patients[168].

2.4 THERAPEUTIC EFFECTS OF TARGETING MITOCHONDRIA IN LIVER DISEASE

Since mitochondria play major roles in liver physiology, it is expectable that variations in their function are associated with hepatic diseases. Alterations in oxidative metabolism (including the TCA cycle, β-oxidation and the ETC), respiratory uncoupling, ROS homeostasis and cell death are all implicated in liver pathology and represent therapeutic targets for the treatment of liver disease[17]. As the mitochondrial pathways and proteins implicated in liver pathogenesis are so numerous, there are at this time many mitochondrial targeting strategies being used in clinical and under research. Hence, in this section, a descriptionof the main types of mitochondrial therapies and some examples of drugs and targets will be provided.

It has been shown that increasing the levels of antioxidants in mitochondria mitigates and prevents the oxidative damage associated to most chronic and acute liver injuries including NASH, fibrosis, cirrhosis, ischemia-reperfusion, cholestasis and alcohol and drug-induced liver injuries[131,169]. For instance, in the animal model of DILI caused by APAP overdose, the administration of the GSH precursor NAC prevents the toxicity of APAP and APAP-associated ROS, restoring mitochondrial function and ameliorating liver injury[170]. In this line, in the animal model of obstructive cholestasis induced by BDL, NAC treatment reduced liver injury and fibrosis[171]. Both in experimental models of NAFLD and in NASH patients, NAC treatment showed a significantly improvement in aminotransferase levels[169,172]. Besides NAC treatment, the overexpression/deletion of genes like NRF2 and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), and others that directly or indirectly regulate mitochondrial function and ROS homeostasis, have also shown protective/deleterious effects in mouse models of DILI, non-alcoholic fatty liver and cholestasis[173,174].

Targeting mitochondria with specific anti- and pro-apoptotic molecules represent an alternative effective treatment for other liver diseases such as HCC and also to prevent organ rejection after liver transplantation. For example, Bcl-2 overexpression reduced the ischemia-reperfusion injury after orthopic liver transplantation in rats[175]. Furthermore, *in vitro* studies where human hepatoma cells were treated with chemotherapeutic drugs like doxorubicin and mitoxantrone revealed that HCC chemotherapy induces the expression of pro-apoptotic proteins like Bax, Apaf-1 and p53 that promote apoptosis via the intrinsic and extrinsic signaling pathways[176].

Targeting mitochondrial β-oxidation, ETC and uncoupling proteins is also considered a possible therapy for the treatment of liver diseases related to obesity and diabetes, like NAFLD[177,178]. However, the treatment of NAFLD is challenging and therapeutic strategies targeting mitochondria are still in very early stages. Two examples of proteins under research are PPARα and sirutin 1 (SIRT1), which have shown anti-steatotic properties *in vivo*. Both PPARα and SIRT1 sustained expression decreased the accumulation of fat in the liver of the hyperphagic obese model of leptin deficiency

ob/ob mouse and methionine-choline deficient diet (MCDD) fed mice by increasing FA β-oxidation[179–181]. Regarding the respiratory uncoupling, one example is the use of 2,4-dinitrophenol (DNP) to treat obesity: DNP promotes weight loss by increasing mitochondrial proton leak, which leads to decreased OXPHOS and rapid stored fat consumption[182].

All these data highlight the importance of mitochondria both in normal liver function and liver disease. To better understand the mechanisms by which mitochondria regulate the initiation and progression of DILI, NAFLD, cholestasis and HCC, experimental models resembling each human disease along with two knockout (KO) mouse models for the mitochondrial proteins prohibitin 1 (PHB1) and methylation-controlled J-protein (MCJ) were used.

2.5 MOUSE MODELS OF ALTERED MITOCHONDRIAL FUNCTION

2.5.1 Increased mitochondrial function: Methylation-controlled J protein knockout mouse (MCJ KO)

Methylation-controlled J protein (MCJ), also known as DnaJC15, is a small protein that belongs to the DnaJ family of co-chaperones. Unlike other DnaJ proteins, MCJ is not soluble since it contains a transmembrane domain and localizes in the inner mitochondrial membrane (IMM). MCJ was first discovered in ovarian cancer cells where it was found to be negatively regulated by methylation of CpG island[183,184]. Moreover, it has been shown that the loss of MCJ associates with chemoresistance in human breast and ovarian cancer cell lines[183,185–187]. The ortholog of human MCJ has been recently identified in mice and it has been shown that is highly expressed in heart, liver and kidney, tissues with active mitochondrial metabolism[188].

Importantly, Mercedes Rincon's group has reported that MCJ interacts with and represses the function of complex I of the ETC, making it the first **endogenous inhibitor of complex I**. They have demonstrated that MCJ deletion *in vivo* results in increased complex I activity, MMP and ATP production, without affecting mitochondrial mass. Additionally, it has been shown that MCJ interferes with the formation of respiratory SC, which facilitate an efficient transfer of electrons and minimize ROS production[188]. On the other hand, MCJ co-chaperone function has also been studied. In this regard, it has been shown that MCJ forms a supercomplex with MAGMAS, major component of the mitochondrial import machinery that interacts with TIM23 (translocase of the inner membrane 23), a pre-protein translocase, promoting the import of proteins into human mitochondria. Furthermore, MCJ has been linked to cell death by recruiting and coupling cyclophilin D to permeability transition[189]. The role of MCJ within the immune system has also been investigated. It has been shown that MCJ is highly expressed in $CD8^+$ T-cells, but not in $CD4^+$ T-cells and B-cells[188], and less in macrophages, in which the transcriptional regulator Ikaros mediates its DNA methylation-independent silencing[190]. In this regard, it has been recently published that $CD8^+$ T-cells lacking MCJ provide superior protection against influenza virus infection due to increased OXPHOS and ATP production, selectively increasing the secretion, but not expression, of interferon-γ[191].

As observed in MCJ KO mice, under physiological conditions, the lack of MCJ does not cause an altered phenotype[188]. However, in conditions that lead to an over-accumulation of fat in the liver, such as fasting and a high-cholesterol diet, MCJ deficiency prevents such accumulation, suggesting that MCJ may also act as a brake of mitochondrial respiration in liver[188]. Moreover, it has been observed that MCJ is essential for the production of TNFα by macrophages in response to infectious insults such as bacteria. Particularly, MCJ-deficient mice have shown resistance against fulminant liver injury upon lipopolysaccharide (LPS) administration[192].

These findings point out the important role of MCJ as an essential negative regulator of mitochondrial metabolism and provide new basis and tools (MCJ KO) for the study and discovery of new mechanisms regulating mitochondrial liver diseases along with therapies to treat them.

2.5.2 Decreased mitochondrial function: Prohibitin-1 knockout mouse (*Phb1* KO)

Prohibitin-1 (PHB1) is a very conserved and ubiquitiously expressed protein involved in multiple cellular pathways depending on its subcellular localization. It is mainly localized in the IMM, where it exerts a chaperone-like function to stabilize newly synthesized mitochondrial proteins and maintain the organization and stability of mitochondrial nucleoids[193,194]. PHB1 is essential for mitochondrial biogenesis in yeast[195]. Furthemore, it regulates the stability of optic atrophy 1 (OPA1), a mitochondrial fusion regulating protein. OPA1 regulates mitochondrial fusion and cristae structure and contributes to ATP synthesis and apoptosis[196]. Importantly, PHB1 is also found in the nucleus where it interacts with retinoblastoma (Rb) and p53 among other proteins and recruits nuclear receptor co-repressor (N-CoR) and histone deacetylase 1 (HDAC1) to induce a change in the transcriptional activity of E2F7 and p53[197,198]. These nuclear effects have been associated with the inhibition of cell cycle progression[195] and the induction of apoptosis[196]. Given the implication of PHB1 in so many vital functions and its reduced expression in methionine adenosyltransferase 1A (MAT1A)-KO mice (animals that develop spontaneously NASH and HCC[199,200]), in the *ob/ob* mice and in obese people at risk of developing NASH[201], a liver-specific *Phb1* KO mouse was generated[202]. This mouse is the first *in vivo* model that allows examination of the consequences of decreased PHB1 expression.

Characterization studies by the laboratory of Shelly C. Lu showed that the liver specific *Phb1* KO mice develop liver injury at a very early age, fibrosis, liver cell dysplasia and HCC[202]. At only 3-weeks of age most *Phb1* KO appear ill and liver injury is evident with marked necrosis and inflammation throughout the liver and 8-fold increase in alanine transaminase (ALT). At this early age there is also bile duct metaplasia and positive staining for preneoplasic and ovall cell markers. Importantly, hepatic mitochondria in the 3-week old *Phb1* KO appear swollen and many have no discernible cristae. Consistent with **impaired mitochondrial function** and oxidative stress, increased lipid peroxidation was observed. Additionally, microarray analysis revealed up/down-regulation in 402/182 genes. Several of these genes are involved in cell growth like H-19, epidermal growth factor receptor 1 (EGFR1) and Cyclin D1 and in fibrogenesis, like many collagen genes. The other regulated genes are associated with many different signaling pathways: angiogenesis, inflammation, phosphoinositide 3-kinase (PI3K), Ras, and vascular endothelial growth factor (VEGF), among others. As *Phb1* KO animals grow older, there is progressive apoptosis and fibrosis. By 14 weeks, hepatic nodules can be seen in some liver sections, by week 20 all mice have multiple liver nodules and between the ages of 35 to 46 weeks, 38% have multifocal HCC[202].

The ability of PHB1 to directly modulate growth was confirmed in mouse AML12 hepatocytes, in which PHB1 knockdown resulted in cyclin D1 increased expression and enhanced proliferation[202]. Also, LPS/D-galactosamine N (GalN) administration to WT and *Phb1* KO mice confirmed that PHB1 deficiency predisposes to liver injury[203]. And finally and importantly, it was been recently observed that the activation of a novel c-Myc-miR27-PHB1 circuitry during obstructive cholestasis inhibits the synthesis of GSH in mice sensitizing the liver to injury[204]. Taken together, these results evidence the importance of PHB1 in maintaining normal liver function and suggest that the fall in its expression contributes to the pathogenesis and progression of liver disease as well as predisposes to HCC. The progression of liver damage over time in *Phb1* KO mice is shown in Figure 2.10.

In the same way that we believe that mitochondrial dysfunction is a major and common event in the pathogenesis of most liver diseases, we believe that alterations in PTMs may be involved in the initiation and progression of different hepatic disorders.

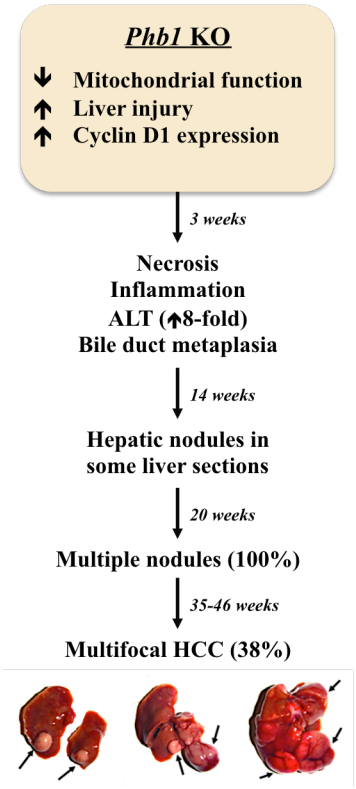

Figure 2.10 *Phb1* KO pathological features. *Phb1* KO mice present mitochondrial dysfunction and clear liver lesions at a very early age. By 4-5 months 100% of animals have multiple nodules and by 8-10 months multifocal HCC.

2.6 POST TRANSLATIONAL MODIFICATIONS IN LIVER DISEASE

PTMs are considered key mechanisms regulating protein homeostasis and function in eukaryotic cells. Moreover, these modifications extend the diversity of the proteome by inducing structural and functional changes in proteins through different mechanisms like covalent binding of functional groups, cleavage of regulatory subunits and degradation of other proteins. Protein PTMs influence enzymatic activities, protein turnover, subcellular localization, protein-protein interactions, DNA repair and cell division, among other processes. Thus, PTMs are essential to maintain normal cellular signaling, metabolism and function[205,206]. The most common modifications include phosphorylation, methylation, acetylation, glycosylation. Because PTMs are necessary for normal physiology, alterations in their pathways have been associated with the development and progression of many diseases[207]. In the liver, PTMs also influence almost all aspects of normal cell biology and aberrant modifications have been linked to different hepatic pathologies. For all these reasons, in the last few years many groups have tried to understand how changes in protein homeostasis may drive pathogenesis of human diseases, providing basis for the discovery of several important therapies.

In here, a brief description of acetylation, ubiquitination and the ubiquitin-like molecule Nedd8 (neural precursor cell-expressed developmentally downregulated-8)-mediated modification, neddylation PTMs and their roles in regulating liver physiology will be provided.

2.6.1 Acetylation

Acetylation is considered one of the major PTMs in the cell and is thought to occur in 80-90% of human proteins[208]. It is involved in important biological processes like cell cycle and apoptosis and consists of the covalent addition of an acetyl group to the N-terminal or lysine residues of proteins. The transfer of an acetyl

group from acetyl-CoA to the N-terminal of proteins is catalyzed by N-acetyltransferases (NATs) and to the ε-amino group of lysine residues by histone acetyletransferases (HATs). Even though lysine acetylation was first discovered in histones it also occurs on non-histone proteins (Figure 2.11). Acetylation modification neutralizes the positive charge of the lysine residue affecting protein function, stability, subcellular localization, interactions with other proteins or DNA, enzymatic activities and its propensity to further modifications[208]. On the other hand, acetylation of histone lysine residues is a major mechanism for gene transcription regulation and is known as the **histone code**. The histone code hypothesizes that the transcription of genetic information encoded in DNA is in part regulated by chemical modifications to histone proteins[209]. Unlike N-terminal acetylation, which is irreversible, lysine acetylation is a reversible process that should be tightly regulated. For this reason there also exist **HDACs** that counteract HATs. While histone acetylation reduces chromosomal condensation and stimulates gene transcription, histone deacetylation increases chromosomal condensation, repressing gene transcription. It should be noted that the balance between acetylation and deacetylation is essential for many important cellular processes and its disruption has been associated with different human diseases[210].

Acetylation imbalance has been reported in a variety of hepatic disorders. For instance, aberrant histone acetylation is known to occur in HCC. It has been observed that the hepatitis B virus X protein induces histone deacetylation of cancer-related genes, promoting HCC development[211]. Similarly, it has been shown that alcohol-induced protein hyperacetylation contributes to the progression of liver disease[212] and that deacetylation predisposes to liver injury by APAP[213]. Furthermore, aberrant acetylation of the nuclear BA receptor Farsenoid X receptor (FXR) has been associated with fatty liver pathogenesis[214] and liver regeneration[215] . And regarding cholestatic disease, it has been observed that BAs stimulate HDACs and co-repressors recruitment, regulating the expression of important genes related to BA metabolism, like *CYP7A1* (Cholesterol 7 alpha-hydroxylase), *SHP* (Small heterodimer partner) and *FXR*[216]. Importantly, HDACs are considered major regulators of pro-fibrogenic and pro-inflammatory cascades (Transforming growth factor beta (TGF-β)/Smads) in the liver[217]. All these findings point out the importance of acetylation in liver physiology and support a central role for this PTM in liver disease. In the last few years HDACs have become promising targets for a wide range of diseases, including hepatic diseases, and several inhibitors that are now being used in clinical have been developed[217,218]. Two of the most known and common used are trichostatin A (TSA) and vorinostat, which maintain normal acetylation cellular status.

2.6.1.1 Histone deacetylases: HDAC4

HDACs are classified in three main classes, I, II and III, based on their size, sequence homology and distinct complexes formation. Class I HDACs are characterized by small size and nuclear localization and include HDAC1, HDAC2, HDAC3 and HDAC8. Class II HDACs are larger and can shuttle between nucleus and cytoplasm and include HDAC4, HDAC5, HDAC6 HDAC7, HDAC9 and HDAC10. HDAC11 is sometimes placed in class IV since it has conserved residues that are shared by both classes I and II[219]. Finally, class III

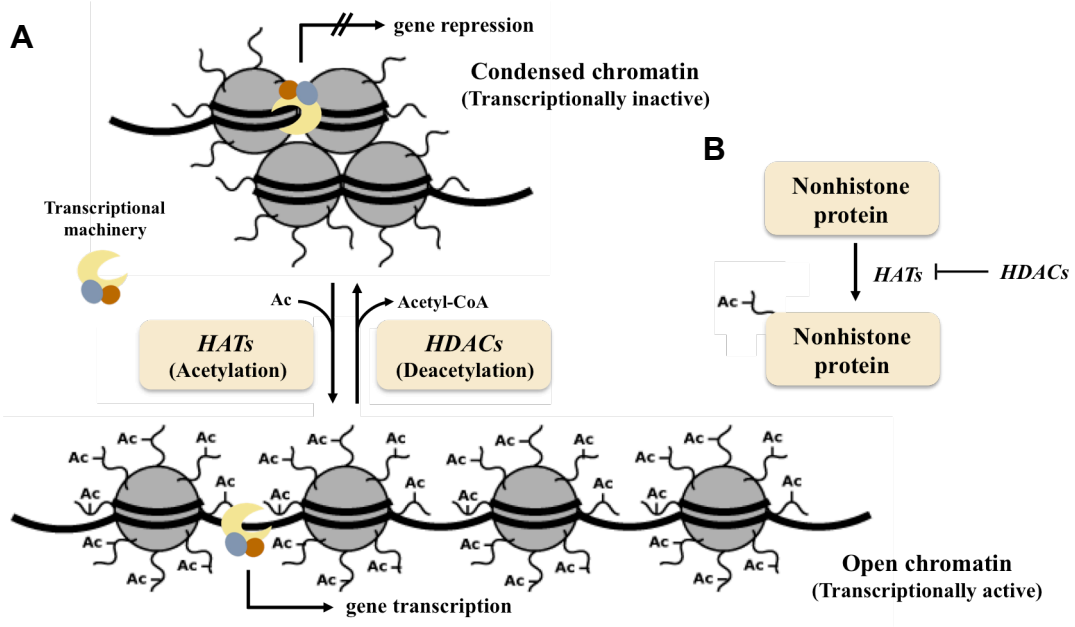

Figure 2.11 Acetylation. Acetylation is the process where an acetyl group is transferred from acetyl-CoA to (A) histone or (B) nonhistone proteins. Histone acetylation and deacetylation, which are catalyzed by HATs and HDACs respectively, are essential processes for gene regulation. When chromatin is acetylated, it is opened and gene transcription is active. On the other hand, low acetylation levels maintain the chromatin condensed and gene transcription is repressed. In the case of nonhistone proteins, acetylation may affect protein function, stability, subcellular localization, interactions with other proteins or DNA, enzymatic activities and its propensity to further modifications.

includes SIRTs, which are NAD-dependent deacetylases that contrarily to the other classes do not contain zinc in their catalytic site[220]. As already mentioned, HDACs owe their name to the fact that lysine acetylation was first discovered in histones and also because these proteins are their main targets. However, a steadily growing number of non-histone proteins are being identified as substrates for HDACs and notably, among these, there are major regulators of cell proliferation, migration and death such as c-Myc, Rb, p53 and NFκB. The acetylation status of the lysine residues is regulated by co-activators and co-repressors transcription factors that contain respectively HAT or HDAC activities. Dysfunction of HATs and HDACs leading to aberrant acetylation of histone and non-histone proteins has been reported in many human cancers[221]. These alterations have been mainly linked to changes in expression of different HDACs more than to structural mutations.

HDAC4 belongs to the class II of HDACs and is expressed in a tissue-specific manner. This enzyme is known to repress the transcription of multiple genes controlling important cellular functions such as cell differentiation, cell cycle, proliferation and apoptosis[222]. HDAC4 does not bind DNA directly, but through transcription factors MEF2C and MEF2D (myocyte enhancer factors 2C and 2D) and multiprotein corepressor complexes such as RbAp48-HDAC3. Importantly, HDAC4 has been broadly associated with neurodegenerative diseases like Huntington's disease and its inactivation induces neuronal cell death[223]. In the last few years it has been shown that HDAC4 has a role in gastric, colorectal and some other cancers where it promotes tumor growth by p21 repression through an Sp1-dependent and p53-independent mechanism[224,225]. It is also known that HDAC4 targets hypoxia inducible factor 1 alpha (HIF-1α) regulating its stability and the expression of target genes in cancer[226]. In this line, HDAC4 overexpression has been associated with higher tumor grade and poor survival in a variety of human cancers and its knockdown has been shown to induce growth arrest in different human solid cancer cell lines by promoting apoptosis, which support a role for this protein in tumor cell survival[227,228].

In the liver, HDAC4 regulates HSC activation and its depletion has shown anti-fibrotic properties *in vivo*[229]. Moreover, it has been observed that the small non-coding RNA molecule, the microRNA miR-22, which is downregulated in liver cancer, promotes HCC progression by increasing HDAC4 expression[230]. On the other hand, it has been seen that following ischemia/reperfusion in hepatocytes, oxidative stress promotes HDAC4 shuttling from the nucleus into the cytosol, reducing its transcriptional activity and enhancing liver damage[231]. Also, in response to the peptide hormone glucagon, HDAC4 is translocated into the nucleus where it binds to gluconeogenic enzymes' promoters and activates the forkhead box O (FoxO) family of transcription factors. Hence, loss of hepatic HDAC4 in mice with type 2 diabetes results in reduced blood glucose and increased glycogen storage, suggesting a potential therapeutic role for HDAC4 in metabolic syndrome[232]. Little is known about the role of HDAC4 in liver disease but altogether these findings demonstrate a strong association between its deregulation and a variety of processes underlying hepatic injury.

2.6.2 Ubiquitination and the proteasome system

The ubiquitin-proteasome system (UPS) is a key regulator of cellular protein homeostasis in mammals. Indeed, several studies have demonstrated that protein degradation assumes equal weight as synthesis in determining intracellular protein content. Ubiquitination is the PTM consisting of the covalent binding of ubiquitin (Ub) molecules to substrate proteins, targeting them for **degradation and recycling**[233]. It is a multistep enzymatic process that is catalyzed by several enzymes: Ub activating ezymes (E1), Ub conjugatin enzymes (E2) and Ub ligases (E3). It should be noted that there also exist deubiquitinases that remove Ub from substrates before entering the proteasome and maintain the pool of free Ub molecules (Figure 2.12). Target proteins can be mono- and poly-ubiquitinated depending on their type and subcellular localization, and may be either activated (being or not being translocated) or tagged for proteasomal degradation. It is known that the attachment of long poly-ubiquitin chains marks substrates for degradation by the **26S proteasome**. Ubiquitination accounts for hundreds of substrates and is crucial in almost every cellular process including differentiation, proliferation, autophagy, apoptosis, DNA repair, intracellular signaling and immune response, among others, and for this reason alterations in its pathway have been associated with almost every disease and disorder[234,235]. Furthermore, aberrant proteasomal degradation may increase the effect of oncoproteins such as c-Myc, Ras and β-Catenin and also reduce the amount of tumor suppressors like p53, since these proteins are also ubiquitination targets. Hence, ubiquitination may also affect the outcome of many diseases.

Protein homeostasis and thus, degradation of specific proteins, is crucial for cell signaling and must be tightly regulated in order to allow cells to respond rapidly and appropriately to environmental changes and undergo specific processes. The regulation of ubiquitination is complicated and is usually controlled by E3 ligases and a wide variety of complexes with ubiquitin ligase activity whose assembly is stimulated by different cellular signals[236]. The most known E3 ligases are anaphase-promoting complex (APC) and the Skp1-Cullin-F-box complex (SCF), which both target substrates for proteasomal degradation. Mdm2 (mouse double minute 2 homolog) is a well-described E3 ligase that regulates p53 ubiquitination and proteasomal degradation. Ubiquitination is also regulated by the activity of the proteasome, which is known to decrease with aging and can be affected by different stimulus and injuries[236].

In the liver, the UPS plays a central role regulating most biological processes like cell cycle and the activity of transcription factors like NFκB[146] and HIF-1α[237], deeply implicated in inflammation and cancer. For this reason, disruptions in the ubiquitin

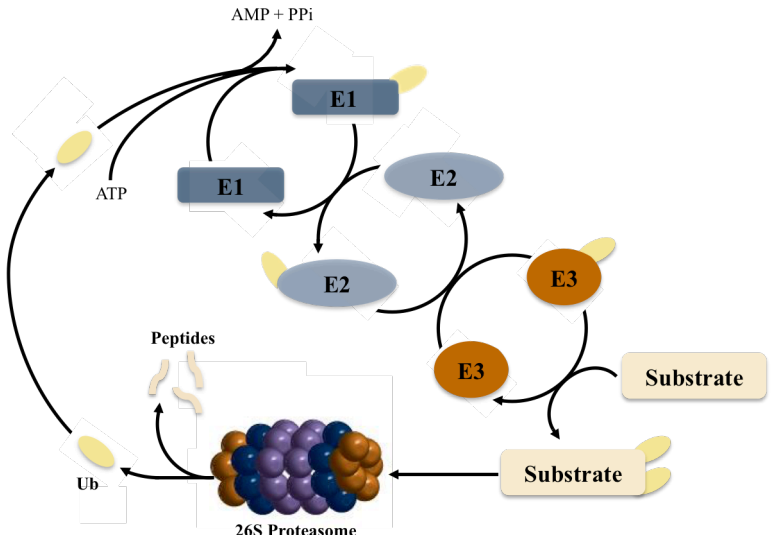

Figure 2.12 Degradation of a protein via the UPS. Initially, Ub activating enzymes (E1) activate Ub in an ATP-requiring reaction. Then, Ub conjugating enzymes (E2) transfer the activated Ub from E1 to Ub protein ligase (E3), which catalyzes the covalent attachment of Ub to the substrate. By successively adding activated Ub molecules poly-ubiquitin chains are generated, which serves as a recognition marker to the proteasome. Finally, tagged protein is degraded by the 26S proteasome complex leading to the release of free and reusable Ub molecules. This last process is mediated by deubiquitinating enzymes.

pathway have been observed in most hepatic disorders and may lead to hepatocellular injury by enhancing proliferation and apoptosis, and impeding a correct inflammatory response of the liver. Increased activities of a variety of E3 ligases have been reported in different liver diseases like Mdm2 in HCC[238] and synoviolin in liver fibrosis[239]. Along with this, reduced proteasome activity has been detected in other conditions like alcohol-induced liver injury, correlating with oxidative stress[240]. Likewise, deregulated proteasome activity has been reported in HCC[241] and NAFLD[242]. In accordance with these findings, the levels of Ub serve as a marker of Mallory bodies in ALD[243], cell injury in NASH[244] and tumor growth in HCC[245]. The regulation of the UPS during cholestasis is not clear but different observation like that BAs increase the stability of SHP by inhibiting its proteasomal degradation[246] and the premature degradation of bile transporters during intrahepatic cholestasis[247] suggests both adapations and deregulation of ubiquitination in cholestatic disease. All these data highlight the implication of ubiquitination in normal liver physiology and demonstrate its aberrant deregulation in liver disease.

Since targeting the UPS is a promising strategy for treating cancer and other diseases, different inhibitors that block the proteasome or specific E3 ligases have been developed. Bortezomib is the most commonly used proteasome inhibitor and has shown significant anti-tumoral effects either alone or in combination with other chemo/radiotherapy agents in HCC[248] and protective effects against DILI by decreasing CYP2E1 expression and activity[249]. Although in most cases targeting the UPS implies its inhibition, in some other cases ubiquitination might be beneficial, like promoting the proteasomal degradation of the oncogenic IκB (inhibitor of kappaB), inhibitor of the essential regulator of inflammation, immunity and apoptosis, NFκB.

2.6.3 The neddylation pathway

Nedd8 is an ubiquitin-like molecule that was first discovered in 1992 and shares 60% identity and 80% homology with Ub[250]. Nedd8 is highly conserved in eukaryotes and is expressed in most tissues where it is mainly localized in the nucleus. It has been demonstrated that Nedd8 plays a vital role in regulating processes such as cell growth, viability and development and hence, alterations in its pathway have also been associated with a variety of human diseases[251]. The binding of Nedd8 molecules to target substrates can affect their **stability**, subcellular localization, conformation and function.

The Nedd8 conjugation pathway is similar to that described for Ub, resulting in the covalent conjugation of a molecule of Nedd8 to a lysine residue of the substrate protein. As shown in Figure 2.13 the neddylation pathway is a 3-step enzymatic cascade that involves the activities of E1 activating enzyme, E2 conjugation enzyme and E3-ligase. Nedd8 precursor form is first processed at the C-terminal glycine residue by a Nedd8 protease into the mature form. Then, Nedd8 is activated by E1 Nedd8-activating enzyme (**NAE1**), which is formed by APP-BP1/Uba3 (APP binding protein 1/ubiquitin-like modifier activating enzyme 3) heterodimer, and transferred to E2 Nedd8-conjugating enzyme (Ubc12, Ube2F). Ultimately, a substrate specific-E3 ligase transfers Nedd8 to the lysine of the substrate[251]. There exist multiple Nedd8 E3 ligases like Mmd2, DCN1 (defective in cullin neddylation 1) and SCF^{FBX011}. All known Nedd8 ligases belong to the RING subclass and can also function as E3 ubiquitin ligases[252]. It should be noted that similarly to ubiquitination, neddylation is reversible through the action of isopeptidases (named deneddylases) such as COP9 signalosome (CSN) and Nedd8 protease 1 (NEDP1), which free the substrate and Nedd8[253]. It has been shown that poly-Nedd8 and Nedd8-Ub chains can be formed *in vitro* but it is unclear whether they have a function *in vivo*. Furthermore, it has been observed that under diverse stress conditions, ubiquitin enzymes like Ube1 (Ub E1 enzyme) participate in the neddylation process[254].

Until recently, the only known substrates of Nedd8 modification were the cullin proteins of the cullin-RING E3 ligases (CRLs), the largest E3 ubiquitin

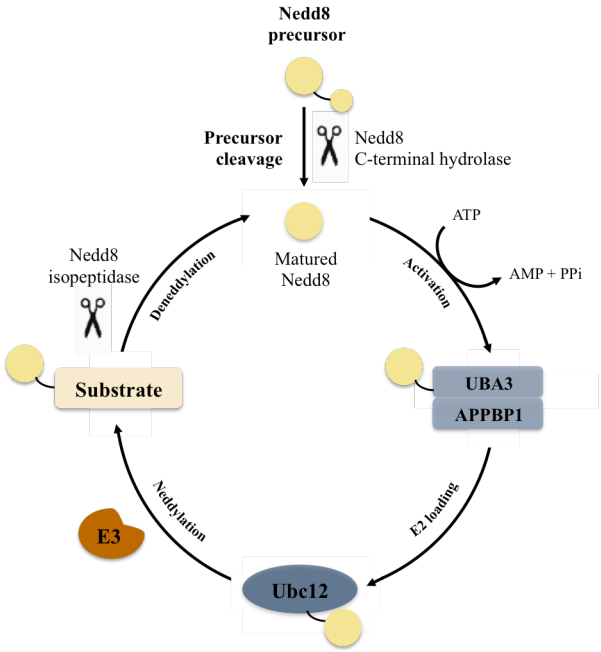

Figure 2.13 Nedd8 conjugation pathway. Nedd8 precursor is cleaved to obtain mature Nedd8, which is activated by NAE1 (E1) and loaded into an E2 enzyme (Ubc12, Ube2F). Then, an E3 ligase conjugates Nedd8 to the substrate. The deneddylating enzymes reverse the process. Adapted from[251].

ligase superfamily that controls the turnover of many proteins such as p21, p27, cyclin D1, β-catenin, c-Jun or NRF2, targeting them for ubiquitination and proteasomal degradation. These enzymes require Nedd8 conjugation onto the cullin subunit to be active and regulate diverse processes, such as transcription, signal transduction, cell-cycle progression and stress responses[252]. Deneddylation by CSN and NEDP1 has the opposite effect on CRLs, thus inactivating their ubiquitination activity. Notably, NEDP1 is highly specific for Nedd8 but is not specific with regard to the modified substrate[255]. Therefore, cullin neddylation and deneddylation are important to maintain the ubiquitination pathway and cellular protein homeostasis. Since numerous substrates of CRLs are involved in cancer, the deregulation of the neddylation pathway may affect their ubiquitination and degradation, altering cell cycle progression, DNA replication, stress response and other processes, and contribute to carcinogenesis[256].

Nowadays, it is well known that Nedd8 conjugates to a broad range of proteins besides cullins. In fact, in the last few years some neddylation targets have been identified: the oncogene Mdm2, the tumor suppressor p53 and its homologue p73, HIF-1α and its regulator VHL (von Hippel-Lindau), L11 ribosomal protein, EGFR and breast cancer-associated protein 3 (BCA3), among others[257–261]. Interestingly, Dr. Xirodimas group demonstrated that Mdm2, E3 ubiquitin ligase and specific inhibitor of p53, also promotes p53 neddylation attenuating its transcriptional activity[259]. In this line, it has been shown that HuR (Hu antigen R), which is highly expressed in HCC and colon metastasis to the liver (revised in section 3.6.5.4), is stabilized by neddylation through Mdm2[262]. These findings have revealed a new mechanism of stabilization of oncogenic drivers in liver cancer and might be very useful for the discovery of new therapeutic agents to treat HCC.

MLN4924 (Millennium Pharmaceuticals Inc.) is a potent neddylation inhibitor that binds to the ATP-binding site in NAE1 and forms an irreversible MLN4924-NAE1 adduct, inhibiting Nedd8 activation[263]. This drug is being now used in phase II clinical trials for certain leukemias and in phase I for some types of lymphomas, myelomas and advanced solid tumors[264,265]. Importantly, MLN4924 has also shown anti-tumoral effects *in vitro* in lung, gastric, cervical, colon, breast and liver cancer cells by inducing DNA rereplication, increased expression of CRL substrates and apoptosis, and *in vivo* in different human tumor xenografts where neddylation inhibition blocked tumor growth[256,266]. So, the neddylation pathway is considered at this time a promising target for treating cancer and its effect in HCC has not been deeply explored.

2.7 MAIN PATHWAYS IMPLICATED IN THE PATHOGENESIS OF LIVER DISEASE

2.7.1 Main pathways implicated in the pathogenesis of DILI

As already commented in section 3.1.1.3, DILI is a leading cause of ALF and liver transplantation in the US and most of Europe. Actually, it is estimated that drugs cause around 60% of all cases of ALF[21,22]. APAP is the drug most frequently involved in DILI (50% of ALF due to drugs) and its metabolism has been broadly studied and used as example of the toxicity of most of the drugs[24]. On the other hand, idiosyncratic drugs cause around 10% of the clinical cases of DILI[26], including cholestatic liver injury, which can present acutely or in the form of chronic liver disease[27]. Whereas drug-induced steatosis is a common event in DILI patients, only a small proportion of patients may develop chronic liver disease[28].

In this section, the main mechanisms underlying APAP-induced DILI will be described and an overview of the experimental mouse model of DILI will be given (Figure 2.14).

2.7.1.1 APAP metabolism: NAPQI formation and GSH depletion

We have already mentioned that the liver is specifically susceptible to drug toxicity due to its role in clearance[6]. APAP is the most widely used painkiller in the world and whereas at therapeutic doses (up to 4 g/day for adults) it is safe, its overdose, accidental or intentional, may lead to ALF. Once in the liver, APAP is metabolized by conjugation with sulfate (20-30%) and glucoronidate (55-60%) and excreted in the urine[23]. However, a small fraction of APAP, up to 10%, is converted by several cytochromes P450 (mainly by CYP2E1) into NAPQI, a highly reactive and toxic metabolite. Although under normal doses of APAP,

NAPQI can be eliminated from the body by GSH conjugation, large doses of APAP saturate the sulfonation reaction and **NAPQI depletes hepatic GSH**[23–25]. In mice, hepatotoxic doses of APAP deplete 90% of GSH both in the cytosol and mitochondria. For this reason, if DILI is detected in early stages, it can be treated with NAC (GSH precursor) preventing significantly liver injury[170]. Importantly, unconjugated NAPQI is able to form covalent bonds with thiol groups of cysteines of proteins and non-proteins, compromising their activity and rapidly inducing cell death. The amount of covalent bonds with intracellular proteins correlates to the degree of hepatotoxicity[267].

2.7.1.2 Mitochondrial dysfunction

That mitochondria are critical targets of APAP has been known for a long time. Indeed, microscopic examinations have shown mitochondrial morphological changes after APAP treatment[267]. Moreover, several studies have described that NAPQI inhibits mitochondrial respiration in isolated primary hepatocytes and liver mitochondria through **complexes I and II,** without affecting complexes III, IV and V[267–269]. It has been suggested that NAPQI can bind covalently complexes I and II and block their activity, but this remains unclear. A recent work where complexes I and II activities were measured revealed that NAPQI can inhibit mitochondrial respiration in a dose-dependent manner, reducing complex I activity up to 50% and complex II activity up to 90%. In addition, ATP biosynthesis was severely impaired under NAPQI-induced complex II inhibition[268]. Although most studies regarding ETC activity agree in the fact that APAP inhibits mitochondrial respiration and ATP synthesis, the mechanisms and ranges of inactivation remain unknown and controversial. Along with the direct inhibition of ETC complexes, the covalent interaction of NAPQI with other mitochondrial proteins and the overproduction of reactive metabolites are important contributors to mitochondrial dysfunction and **ATP depletion** in APAP hepatotoxicity[23–25].

2.7.1.3 ROS generation and cell death

Hepatocytes, as most mammalian cells, have a highly reducing intracellular environment (GSH:GSSG ≥ 100:1). Mitochondria contain a different pool of GSH and are thought to have an even more reducing environment than cytoplasm[270]. Thus, GSH depletion may deeply impact cellular and mitochondrial redox status and alter protein function[24,267]. Moreover, since GSH has a major role detoxifying H_2O_2, its depletion also affects ROS generation by mitochondria. In accordance with this, a significant increase in H_2O_2 has been detected *in vitro* after 1h of APAP treatment[271]. Importantly, H_2O_2 can also react with protein thiol groups, producing disulfide bond formation, sulfenic acid and other redox changes that disturb protein function[272]. Besides GSH depletion, the impairment of OXPHOS by NAPQI direct binding to ETC proteins represents another important source of ROS in DILI. The inhibition of complexes I and II by APAP may give to the formation of superoxide radical and other ROS due to leakage of electrons from the chain[268,273]. In addition, it has been shown that APAP causes the generation of NO in the liver by the upregulation of inducible and and endotelial nitric oxide synthases (iNOS and eNOS). The formed NO can react with superoxide and form peroxinitrite, a strong oxidant[274]. Brief, APAP hepatotoxicity is associated with the generation of high levels of **ROS** and **RNS t**hat largely contribute to cell death and liver injury.

Hepatocyte death following APAP overdose is a critical event in the clinical manifestation of liver damage. Glutamate dehydrogenase (GLDH) serum levels serve, for instance, to detect liver diseases caused predominantly by necrosis, like DILI[23,275]. Hepatotoxic doses of APAP cause necrotic cell death, even though apoptosis has also been observed in animal and *in vitro* models of DILI. In those experiments, very little caspase cleavage was observed and caspase inhibitors were not able to prevent APAP-induced liver injury[276]. Hence, it was confirmed that necrosis is the primary mode of hepatocyte death in DILI. Interestingly, mitochondrial membrane permeabilization also occurs during APAP toxicity and is considered an important event in DILI. NAPQI may induce **MPTP opening** directly by binding to thiol groups of the pore or through GSH depletion and enhanced ROS. Bcl-2 family members have also been associated with MPTP in APAP-induced liver injury[277].

It is well known that necrosis is more associated to inflammation than apoptosis. This is because necrosis involves the rupture of the plasma membrane and the release of pro-inflammatory factors that may activate the immune system and enhance hepatic injury. It has been described that KCs and other macrophages are activated during APAP overdose but its role continues to be debated. After APAP treatment, these cells produce and release cytokines such as TNFα and interleukin 1 beta (IL-1β), which are pro-inflammatory but also the anti-inflammatory IL-10[278,279]. In this line, it has been shown that the depletion of hepatic NKs (natural killers) before APAP administration decreased hepatocellular necrosis, implicating these cells in the progression of the disease[280]. Notably, necrosis may also activate or inhibit key signaling pathways that regulate cell death and survival.

2.7.1.4 Main signal transduction pathways involved in DILI

After cell injury, many signaling pathways implicated in energy metabolism, cell repair and cell death can be activated and alter the degree of cellular necrosis. For instance, the **JNK** signaling pathway plays a major role in APAP hepatotoxicity regulating cell death and liver injury. It has been observed both *in vitro* and *in vivo* that JNK inhibition (with the inhibitor SP600125) protects against APAP toxicity[267]. Hence, besides GSH depletion and ROS overproduction, intracellular signals that trigger hepatocyte death by “programmed necrosis” are crucial events in DILI. The Blc-2 family of proteins is also involved in APAP toxicity and can be activated upon JNK phosphorylation and translocation to

mitochondria. Bax activation and translocation along with Bcl-xL inactivation have been documented in APAP treatment *in vivo* and are suppressed by JNK inhibition[281]. Similarly, the translocation of JNK to mitochondria may induce the release of Cyt *c* and Smac/DIABLO into the cytosol leading to apoptosis. It has been described that JNK is phosphorylated by apoptosis signaling-regulating kinase 1 (ASK-1), which is self-activated after its dissociation from thioredoxin, whose thiol groups are oxidized by H_2O_2 or other oxidants upon APAP treatment. Likewise, APAP can cause redox changes that sensitize hepatocytes to TNFα and other cytokines. It has been indeed observed that low doses of APAP inhibit NFκB, which is essential in protecting hepatocytes against TNFα cytotoxic effects[282].

Finally, but not less important, is the activation of the **NRF2-ARE** signaling pathway by APAP. The redox changes produced by NAPQI lead to the oxidation of the thiol groups of Keap1 (in charge of retaining NRF2 in the cytoplasm) and the translocation of NRF2 into the nucleus, where it binds ARE and induces the expression of a battery of antioxidant genes including GCL (glutamylcysteine ligase), rate-limiting enzyme in GSH synthesis, HO-1 (heme oxygenase) and catalase[173,283]. The activation of this pathway constitutes an important antioxidant response against APAP hepatotoxicity. Other pathways may be activated or inhibited during APAP overdose as a consequence of ROS/RNS damage to DNA, lipids or other molecules.

2.7.1.5 APAP overdose mouse model of DILI

The reason why APAP is the most used model for studying DILI is because unlike many other drugs, it causes liver injury in animals in a dose-dependent manner[284]. As it occurs in human DILI, hepatotoxic doses of APAP (>250mg/kg) cause rapidly severe liver injury in mice. APAP intraperitoneal injection leads within hours to GSH depletion, protein adducts formation, centrilobular necrosis, high levels of serum ALT and AST (aspartate aminotransferase), and mitochondrial dysfunction (low ATP levels). Moreover, it has been shown that APAP overdose in murines produces overproduction of ROS and RNS and the activation of the JNK pathway along with a strong immune response (infiltration of neutrophils and NKs and activation of KCs and other macrophages) and hepatic inflammation (production of pro-inflammatory cytokines like TNFα and IL-1β). In this model, more than 48h of APAP overdose can be lethal for mice[90,267,285].

2.7.2 Main pathways implicated in the pathogenesis of NAFLD

As mentioned in section 3.1.1.1, NAFLD is the most common chronic liver disease in Western world and is becoming a major global health problem[31,32]. Moreover, NAFLD encompasses a wide spectrum of clinical and histopathological disorders ranging from simple accumulation of lipids within hepatocytes

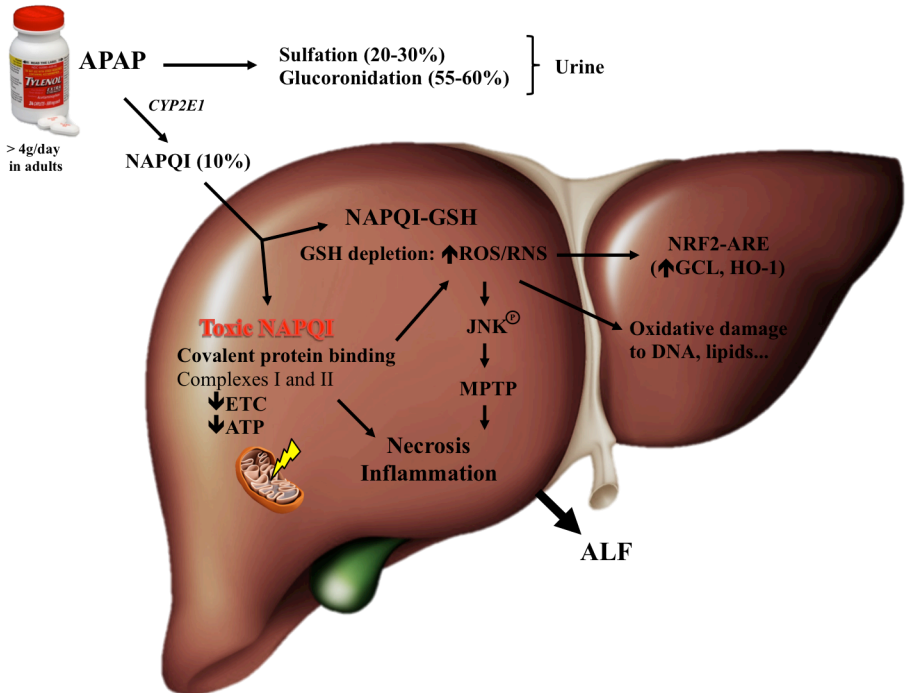

Figure 2.14 Main pathways implicated in the pathogenesis of DILI. Although 90% of APAP that enters the body is conjugated to sulfate and glucoronidate and excreted in the urine, there is a 10% that is converted into the reactive and toxic metabolite NAPQI. To be eliminated, NAPQI conjugates to GSH. In cases of APAP overdose, GSH is depleted and NAPQI binds covalently to proteins including complexes I and II of the ETC, inhibiting their activity. NAPQI impairs OXPHOS and ATP production. Although the antioxidant system (NRF2-ARE activation) tries to alleviate oxidative stress, extremely high levels of ROS/RNS promote JNK activation, oxidation of DNA, lipids and other proteins and the opening of MTPT. Altogether, these events lead to hepatic necrosis with the subsequent inflammation, and may progress, when not treated, to ALF.

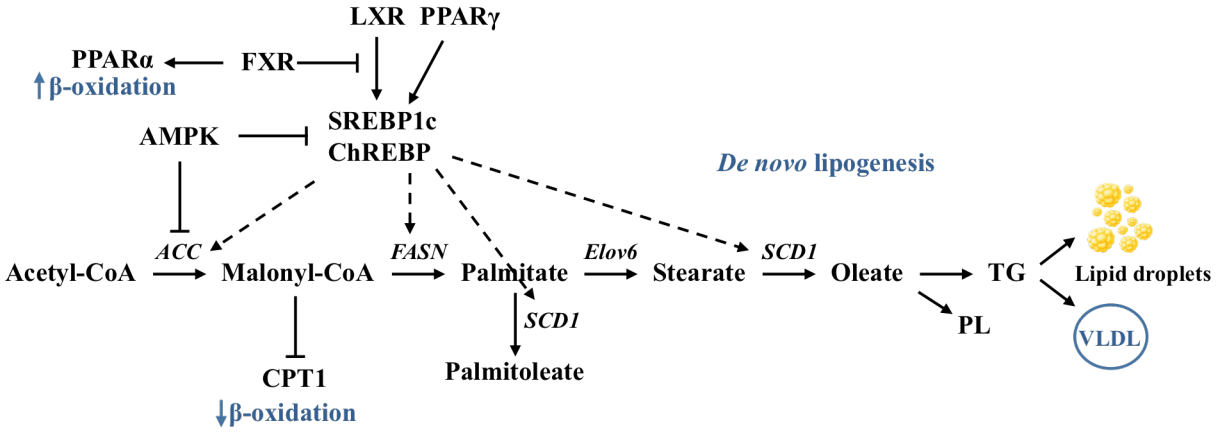

Figure 2.15 *De novo* lipogenesis. *De novo lipogenesis* is the process by which fats are synthesized from simple recursors and is ehanced in NAFLD. A variety of transcriptor factors like SREBP1c, ChREBP FXR and PPARγ, which are activated by insulin and glucose, induce the expression of genes that participate in the process like ACC, FASN and SCD1. Notably, increased *de novo* lipogenesis inhibits β-oxidation through CPT1. On the other hand, FXR negatively regulates the process by inhibiting LXR and its downstream targets and also promotes β-oxidation. AMPK also inhibits the process by inactivating important proteins such as ACC, SREBP and ChREBP.

(steatosis), to steatosis with inflammation (steatohepatitis or NASH), fibrosis, cirrhosis and ultimately HCC[13,34].

In this part, the main known mechanisms underlying NAFLD will be reviewed and a description of the experimental mouse model of NAFLD used will be given (Figure 2.16).

2.7.2.1 Alterations in hepatic lipid homeostasis

The pathogenesis of NAFLD and its evolution to fibrosis and chronic liver disease is still unclear and the **two-hits model** remains the leading hypothesis. The **"first hit"** consists on the accumulation of fat in hepatocytes, normally as macrovesicular intracytoplasmic lipid droplets[35]. Although steatosis is mainly caused by TGs accumulation, it can also be produced by the accumulation of other lipids such as FFAs, cholesterol esters (CE), free cholesterol (FC), ceramides, phospholipids (PLs) and diacylglycerol (DG)[286]. This first hit occurs due to metabolic alterations that compromise lipid homeostasis including: 1) increased FA uptake, 2) enhanced hepatic FA synthesis *de novo*, 3) reduced capacity of FA β-oxidation and 4) impaired secretion of hepatic TG as very low-density proteins (VLDL)[174,287,288]. During NAFLD, the first hit enhances the vulnerability of the liver to the **"second hit"**, which comprises many factors that promote liver damage, inflammation and fibrosis. The second hit is multifactorial and consists mainly of oxidative stress with subsequent lipid peroxidation, release of inflammatory cytokines and adipokines, and mitochondrial dysfunction. Notably, oxidative stress is considered the onset of the progression from steatosis to NASH[289].

a) Increased fatty acid uptake and *de novo* lipogenesis

Hepatic ***de novo* lipogenesis** (DNL) has been found significantly increased in NAFLD patients (it is not suppressed even during fasting) and experimental models of non-alcoholic fatty liver[287,290], probably due to a hyperinsulemic situation[291]. Nonetheless, the composition of **the diet** is considered another important factor promoting DNL, as it is usually rich in simple carbohydrates, which are easily converted to FA, and fructose, major activator of DNL. In addition, since around 15% of FAs proceed directly from the diet, this value can increase in diets where more that 30% of the caloric support is in form of fat[292,293]. Besides the diet and DNL, **the white adipose tissue** represents a major source of FAs in the body. In fact, its physiological role is to supply energy (as lipids) to peripheral tissues when required, like during fasting. Under these circumstances, stored TGs are hydrolyzed and released as FFAs (process known as lipolysis). This process is also regulated by insulin and is enhanced during obesity and insulin resistance[287].

At molecular level, DNL is regulated mainly by the transcription factors sterol regulatory element-binding protein-1 (**SREBP-1**) and carbohydrate response element-binding protein (**ChREBP**)[294] (Figure 2.15). SREBP-1 is present in different isoforms whose deregulation leads to hepatic lipid accumulation: SREBP-1c is the isoform responsible for DNL activation and is regulated by insulin and SREBP2 regulates cholesterol homeostasis. On the other hand, ChREBP, which is activated by glucose, also enhances DNL and provides substrates for TG and FFA synthesis. These transcription factors induce the expression of proteins that are directly implicated in DNL like acetyl-CoA carboxilase (**ACC**), FA synthase (**FASN**) and stearoyl-CoA desaturase-1 (**SCD1**). ACC catalyzes the first step of the process: the conversion of acetyl-CoA into malonyl-CoA. FASN synthesizes palmitate (using NADPH as a cofactor) from malonyl-CoA, and finally, SCD1 catalyzes the desaturation of palmitate to palmitoleate (it also desaturates stearate to oleate). Importantly, the energy sensor AMP-activated protein kinase (AMPK) inhibits this pathway by ACC, SREBP-

1c and ChREBP phosphorylation and inactivation. **PPARγ**, which is highly expressed in adipose tissue and regulates adipocyte differentiation and fat storage; **LXR** (liver X receptor), glucose sensor and direct controller of lipogenic genes (ACC, *FAS*, and *SCD1*); and **FXR**, which regulates genes involved in glucose, lipid and BA metabolism, are other relevant transcription factors that upon activation promote DNL[295–297].

b) Impaired fatty acid β-oxidation

Once in the hepatocytes, FFA can undergo β-oxidation in mitochondria to produce energy in the form of ATP. Alterations in this pathway and inefficient β-oxidation have been documented in NAFLD and are considered an important mechanism underlying steatosis[287]. Several studies have revealed an inhibitory effect of fructose (artificial sweetener widely used in soft drinks) on FA β-oxidation[298].

PPARα, which is highly expressed in the liver, is the main transcription factor regulating hepatic β-oxidation[299,300]. Under energy-demanding conditions, its activation promotes FA uptake and catabolism by upregulating the expression of genes involved in FA binding, transport and β-oxidation[301]. In NAFLD, ineffective PPARα sensing blocks FA oxidation, which largely contributes to hepatic lipid accumulation. **CPT1,** already described as the main mediator of long-chain FAs (LCFAs) import into mitochondrial matrix for β-oxidation, is one of the targets of PPARα and a key rate-limiting enzyme in the β-oxidation process. Importantly, CPT1 can be inhibited by malonyl-CoA, the first intermediate of FA synthesis (Figure 2.15). Hence, in NAFLD, the enhanced DNL negatively regulates mitochondrial β-oxidation deriving FA oxidation to other oxidative pathways in other subcellular organelles[287,302]. Unlike in DNL, AMPK promotes β-oxidation through direct binding to PPARα ligand-binding domain.

Although decreased mitochondrial β-oxidation is considered a major event in NAFLD pathogenesis, its increase to prevent hepatic fat accumulation has also been documented[303,304].

c) VLDL secretion

As part of fat metabolism, lipids can be secreted from the liver into blood (mainly to extrahepatic tissues) as VLDL. VLDL are macromolecular complexes formed by a core of TGs and CEs surrounded mainly by alipoprotein B (apoB), FC and PLs, that are synthesized in the liver in response to nutrients and hormones[2,305].

Despite the fact that the overproduction of VLDL is a hallmark of NAFLD, reduced VLDL secretion has also been reported[288,306]. In the liver, both hyperinsulinemia and hyperglycemia (features of obese and metabolic syndrome) promote the synthesis of FAs and cholesterol, which results in increased TGs synthesis and VLDL secretion. However, TGs synthesis prevails over its secretion leading to steatosis[288]. On the other hand, prolonged oxidative stress and inflammation (features of NASH) may lead to the degradation of apoB 100 (liver-specific lipoprotein) and impaired VLDL assembly and secretion, increasing lipid accumulation and worsening liver damage[307].

2.7.2.2 Insulin resistance

Insulin resistance (IR) is defined as the pathological condition in which cells do not respond appropriately to insulin and there is a lack of its downstream signaling. Although the association between NAFLD and IR has been deeply described and studied, if NAFLD is the cause, or the consequence, or both, of IR is still unknown. IR is almost universally found in NAFLD and it is clear that plays an important role in the pathogenesis of the disease[303,308]. For instance and as mentioned above, IR promotes **white adipose tissue lipolysis** and uncontrolled release of FFAs into circulation, which may further enter the liver and accumulate. In addition, SREBP-1c is overactivated in hyperinsulinemia and triggers the synthesis of FAs through DNL. In this line, several animal studies have reported a direct relationship between IR and the degree of steatosis[309]. Also, defects in insulin signaling pathways such as the phosphorylation of the insulin receptor substrate (IRS), impaired activation of AKT2 and the inactivation of glycogen synthase kinase-3 (GSK-3) have been proposed to enhance steatosis[310]. Importantly, the accumulation of lipids itself can increase IR resulting in defective lipid regulation and further accumulation. Altogether, these data evidence the close relationship between IR and NAFLD pathogenesis.

2.7.2.3 Mitochondrial dysfunction

Multiple studies have associated alterations in both mitochondrial function (ETC and β-oxidation) and structure (mtDNA depletion and morphological changes) with the development of NAFLD[91,311]. However, whether mitochondrial dysfunction is a key factor in NAFLD pathogenesis or the consequence of impaired lipid metabolism is still uncertain[309]. Nevertheless, that in hepatocytes excess lipids can affect directly mitochondrial function and lead to their failure is well known. During steatosis, mitochondria (along with peroxisomes) may not be able to handle the high lipid flux and collapse. At this point, the defective respiratory oxidation leads to the production of lipid toxic metabolites and ROS that worsen mitochondrial and hepatocyte injury and activate inflammatory pathways (KC and HSCs) that participate in the progression of the disease[312].

Nowadays, there is no precise explanation for the **progressive reduction of the ETC activity** during NAFLD. It has been observed that some ETC complexes (i.e cytochrome *c* oxidase) are susceptible to ROS and RNS toxicity. Furthermore, TNF-α and KC-derived interferons are able to impair ETC activity, likely by inducing HIF-1α and mtDNA damage. Indeed, it has been demonstrated that mitochondrial dysfunction correlates with TNF-α levels in obesity and IR. Also,

FAs intermediates can inhibit enzymes involved in ETC activity and OXPHOS and activate JNK, which may trigger apoptosis. Finally, reduced adiponectin and increased activity of FoxO1 transcription factor are other mechanisms that might contribute to decline ETC activity during NAFLD[91,178]. Even though there are some discrepancies about mitochondrial dysfunction in NAFLD, impaired ETC activity is a frequent event.

2.7.2.4 Lipotoxicity, oxidative stress and inflammation

Hepatic lipotoxicity plays an important role in NAFLD, but nonetheless, its exact mechanism remains unclear. During steatosis, in parallel with the accumulation of **TGs**, lipotoxic metabolites of **FAs**, such as **DGs**, ceramides and lysophosphatidyl choline are generated, producing oxidative/ER stress, mitochondrial dysfunction and hepatocyte death[313]. In the liver, more than the quantity of FAs, is the type of FA which determines the grade of the lipotoxic effect: decreased monounsaturated/saturated FA (MUFA/SFA) ratio induces apoptosis and liver injury, as reported in the MCDD mouse model of NASH. Importantly, toxic FFAs upregulate the expression of death receptors like Fas and DR5 sensitizing hepatocytes to apoptosis. Both Fas and DR5 expression is increased in the livers of NASH patients[286].

The overproduction of free radicals (both ROS and RNS) is a leading event in the pathogenesis of NAFLD and its evolution to NASH, cirrhosis and HCC. Increased generation of ROS and reduced antioxidant defenses have been observed in human and experimental mouse models of NAFLD[312]. Enhanced peroxisomal and mitochondrial FA β-oxidation, overexpression of CYP2E1 (ROS-producing enzyme highly expressed in the hepatic tissue), accumulation of mtDNA mutations and lipotoxicity are some events that contribute largely to oxidative liver injury in NAFLD[314]. Additionally, impaired mitochondrial respiration, **low GSH** levels and reduced expression of the antioxidant enzymes GPx and SOD have also been reported. Furthermore, the high expression of iNOS as a consequence of TNFα production by KCs, represents a significant source of RNS and so, peroxynitrite, which has detrimental effects on mitochondrial function[178].

Together with lipotoxicity and oxidative stress, hepatic inflammation is a major aspect in NAFLD and its chronicity a key event for the progression of the disease. In fact, several studies have shown evidences of the central role of **cytokines** in NAFLD pathogenesis, both in human and experimental models[35]. During the first and second hits, a variety of pro-inflammatory molecules and adiponectins are released. Remarkably, oxidative stress can directly activate the immune and inflammatory pathways: hepatic oxidative/ER stress along with lipid peroxidation can activate the transcription of proinflammatory cytokines through NFκB and AP-1. Moreover, elevated serum FFAs and visceral adipose tissue, both hallmarks of NAFLD, may also promote inflammation by activation of NFκB and secretion of pro-inflammatory cytokines like TNFα, IL-6 and CCL2 (C-C motif chemokine ligand 2). KCs can release cytokines, chemokines, ROS and NO upon activation by toxic lipids[313,315]. Despite being considered part of the second hit, inflammation may also lead to lipid accumulation and therefore precede steatosis in NASH. In point of fact, NASH patients might present much or any steatosis[316].

Lipotoxicity, oxidative stress and inflammation are all associated with each other and together drive the progression of NAFLD.

2.7.2.5 Progression to fibrosis, cirrhosis and HCC

Not all NAFLD patients are equally susceptible to develop chronic liver injury and liver cancer. Even though most of them present obesity, diabetes and hyperglycemia, there is an important **genetic susceptibility** that determines the severity of the disease and its progression from steatosis to NASH, fibrosis, cirrhosis and HCC[317–319]. As already commented, not all steatotic patients develop NASH: around 20% of NASH patients reach cirrhosis and 4-27% of them HCC. Moreover, those patients that evolve to more complicated conditions do so in widely varying times.

Multiple studies have suggested genetic factors as the main determinants of NAFLD outcome. For instance, genetic polymorphisms of CYP2E1, TNFα, or IL-10 promoter regions have been documented in NAFLD. Whereas these genetic factors are unknown, it has been suggested that they implicate alterations in genes associated with IR and codifying proteins involved in hepatic lipid metabolism[320]. Likewise, oxidative stress plays a pivotal role in the progression of the disease. Some data have associated the oxidative stress immune response (the presence of oxidative stress-related antibodies) with advanced fibrosis and cirrhosis. In the last years, the activation of KCs and recruitment of other macrophages into damaged liver have been identified as important events for disease progression[312].

Hepatocyte death and inflammation lead to the **activation of HSCs**, which is a key event in hepatic fibrogenesis. Upon activation, proliferative and fibrogenic HSCs synthesize collagen and other components of the extracellular matrix (**ECM**) that are deposited to enclose the injury. The generation and expansion of fibrous tissue within the liver determine the degree of fibrosis[321]. Besides HSCs activation, hepatocyte injury and death is considered a mitogenic stimulus that promotes cell proliferation. These new cells, whose proliferation increases along the process, contain less fat as they proliferate and contribute to the distortion of the liver structure and the formation of **nodules**. Sustained hepatocellular proliferation together with the increasing fibrosis contribute in great measure to liver cirrhosis development. As just discussed, this progression may be influenced by many factors like the amount of fat, the degree of inflammation and other genetic and environmental aspects. This aberrant proliferation can evolve with time, through still unclear

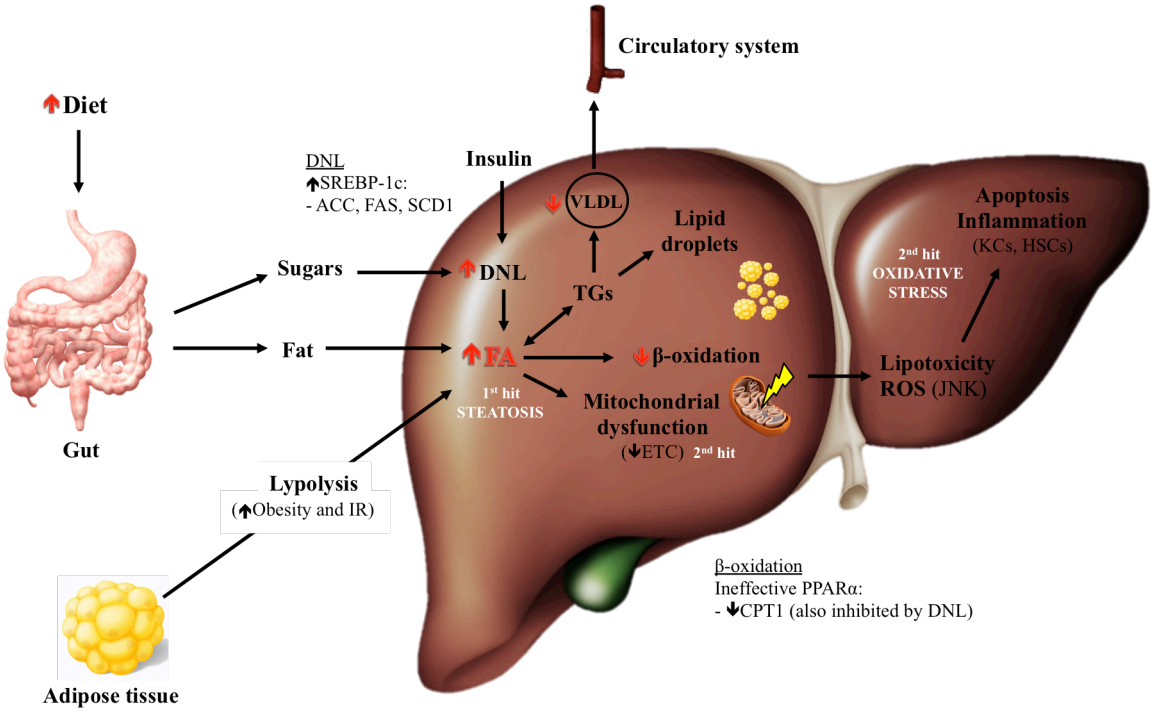

Figure 2.16 Main pathways implicated in the pathogenesis of NAFLD. According to the "two-hits model" of NASH pathogenesis, the "first hit" in NAFLD consists on the accumulation of fat in the hepatocytes, as lipid droplets (steatosis). This first hit occurs due to increased uptake of FA (either from the diet or the adipose tissue), enhanced DNL, which is stimulated by insulin, and impaired β-oxidation and VLDL secretion. During steatosis mitochondria collapse leading to defective respiratory oxidation and overproduction of ROS and lipid toxic metabolites. At this point, the first hit has enhanced the vulnerability of the liver to the "second hit", which comprises other factors (oxidative stress, lipid peroxidation, release of inflammatory cytokines and adipokines, mitochondrial dysfunction) that promote liver damage, inflammation and fibrosis. Hepatocyte death and inflammation lead to HSCs activation and the synthesis of ECM, leading to fibrosis/cirrhosis development. Not all patients are equally susceptible to develop chronic liver injury and cancer but the progression to cirrhosis and HCC is frequent.

mechanisms, to HCC, the end stage of the disease[322].

2.7.2.6 MCDD mouse model of NAFLD

The MCDD is a broadly used mouse model of NAFLD. This model is particularly important for understanding the pathogenesis of the disease since a short-term administration of the diet induces NASH and its prolongation leads to liver cirrhosis and HCC[323]. Notably, MCDD rapidly induces steatosis, focal inflammation, necrosis, fibrosis, elevated aminotransferases and histological changes in the liver, features of NAFLD. Steatosis is induced by 2-4 weeks and shortly thereafter it progresses to inflammation and fibrosis[324,325]. However, unlike human, mice fed with a MCDD lose weight and do not become insulin resistant[326]. The lack of methionine and choline, both critical components of the diet and precursors of S-adenosylmethionine (SAMe) and phosphatidylcholine (PC), compromises many processes including cellular methylation, the redox antioxidant system and VLDL secretion[314]. Increased hepatic lipogenesis (via SREBP-1c), FA uptake (via lipolysis), lipotoxicity and oxidative/ER stress along with reduced β-oxidation have been described in this model[327]. Furthermore, mitochondrial dysfunction, elevated cytokines and the infiltration of lymphocytes and neutrophils in necroinflammatory foci also occur during MCDD and contribute to liver injury[328].

2.7.3. Main pathways implicated in the pathogenesis of cholestatic liver diseases

As described in section 2.1.2.2, cholestatic liver diseases are characterized by the **accumulation of potentially toxic BAs in the liver**[42,43]. Even though the causes for BAs retention in the liver are diverse, once the cholestatic condition has been produced, the progression of cholestatic diseases is similar consisting of hepatocyte death, inflammation and scarring of the liver[45].

PBC, BAT and ALS are common conditions of obstructive cholestasis where bile ducts are progressively destroyed (PBC and BAT), abnormally narrow or even absent (BAT and ALS)[12,54,55]. While PBC affects mostly adults and leads to progressive cholestasis, BAT and ALS are childhood diseases with a more severe cholestatic condition. The prevalence of cholestatic diseases is not high but one third of patients with many disorders still do not respond to the treatment and suffer complications, requiring liver transplant[58–60].

In this part, major mechanisms underlying cholestatic liver diseases will be revised (Figure 2.18)

and the BDL mouse model of obstructive cholestasis will be described.

2.7.3.1 Biliary epithelial cells damage

The hallmark of PBC is the presence of **AMAs** in serum, which are detected in 95% of patients. Even though genetic and environmental factors are considered important in the predisponibility to develop PBC, a critical and unique feature of this disease is the 98% specificity of AMAs targeting biliary epithelial cells (BECs) that line the small and medium intrahepatic bile ducts[56]. The main targets of AMAs are enzymes belonging to the family of 2-oxo-acid dehydrogenase complexes including the pyruvate dehydrogenase complex (PDC-E2). These target antigens are located within the matrix of the IMM and catalyze the oxidative decarboxylation of keto-acid substrates. Remarkably, the expression of these proteins is particularly high in BECs, which explains the predilection of AMAs for small and medium bile duct destruction in PBC[329]. At this point, destroyed bile ducts try (ineffectively) to regenerate but it results in obstructive cholestasis and the accumulation of toxic BAs and other metabolites in the liver, which further enhance liver damage creating a vicious cycle that may lead ultimately to cirrhosis[56].

In BAT, bile ducts are abnormally narrow, blocked or even absent. In some cases bile ducts are damaged by the **immune system** in response to viruses or toxins and in others they did not form properly during pregnancy. In early stages of the disease the extrahepatic bile duct is affected but in later stages intrahepatic ducts are also damaged[330]. In ALS, **mutations** in *JAG1* and *NOTCH2* genes cause abnormalities in the formation of bile ducts, which can also be absent. Like it occurs in PBC, malfunction of bile ducts results in obstructive cholestasis and the accumulation of toxic BAs in the liver, resulting in scarring of the liver and loss of liver function[55].

2.7.3.2 BAs toxicity

The accumulation of toxic BAs is a major event in cholestatic liver disease pathogenesis and the main cause of cholestatic liver injury[42]. BAs are **hydrophobic salts** that constitute the bile with essential biological functions. They are synthesized from cholesterol in the liver, specifically in the cytosol, ER, mitochondria and peroxisomes of hepatocytes and ductal cells lining the hepatic ducts, and are responsible for the solubilization of dietary fats, fat-soluble vitamins and other essential nutrients and their delivery to the liver after being absorbed by the small intestine[331]. For this reason and because there exist many toxic BA intermediates, BA metabolism and its hepatic pool must be tightly regulated. Altered BA metabolism and defects in associated genes have been reported in human cholestasis. Mutations in BA transporter genes like BSEP (bile salt export pump), MDR3 (multiple drug resistance 3) and deficiency in enzymes involved in the synthesis of BA like CYP7A1, are some examples of disorders causing cholestatic liver diseases[332].

Experimentally, BAs are known to cause injury to isolated and cultured hepatocytes and also to the whole liver, but the mechanisms underlying this toxicity are not fully understood. As observed in mouse models and *in vitro* experiments using human hepatocytes and considering that BAs damage cellular membranes via **detergent-like action**, it is thought that their hydrophobicity correlates with their toxicity[333]. Whereas hydrophobic BAs may induce, in a time and dose-dependent manner, apoptosis in hepatocytes, hydrophilic BAs enhance intracellular cyclic adenosine monophosphate (cAMP) thus activating the MAPK and PI3K pathways in order to protect hepatocytes from cell death[334]. Particularly, high concentrations of BAs such as litocholic (LCA), deoxycholic (DCA), chenodeoxycholic (CDCA), glycochenodeoxycholic (GCDCA) and taurochenodeoxycholic (TCDCA) have exhibited hepatotoxic effects.

a) Mitochondrial dysfunction and oxidative stress

Oxidative stress is another important event during cholestatic liver injury both in animals and humans[92,126]. BAs promote ROS generation and lipid, protein and DNA **oxidative damage**, which lead to hepatocyte death. On the other hand, BAs are able to activate KCs to generate ROS that further contribute to liver injury[335]. It has been suggested that hepatic mitochondria are a major source of ROS during BAs toxicity. In fact, it has been shown that BAs impair mitochondrial respiration through the inhibition of the ETC **complexes I, III and IV** (complex II is not affected), which may further give rise to the formation of superoxide radical and other ROS due to leakage of electrons from the chain[336]. Additionally, BAs are able to reduce MMP and enhance the state 4 respiration (uncoupling) by increasing the permeability of mitochondria to protons[337]. These last effects are mainly attributed to the disruption of mitochondrial membranes due to direct interactions of BAs with their structure.

Hydrophobic BAs can also stimulate oxidative damage by the **depletion of antioxidant defenses**, including cytoplasmic and mitochondrial GSH and ubiquinone, which participates in the ETC. Additionally, the induction of the MPTP opening by BAs largely contributes to the generation of ROS, as seen in rat hepatocytes and isolated mitochondria[335]. In accordance with this, antioxidants have shown protective effects against BAs hepatotoxicity by mitochondrial membrane depolarization inhibition. Finally, it has been shown that during obstructive cholestasis the mtDNA copy number and the synthesis of mitochondrial proteins are altered due to impaired translation of mitochondrial mRNA, as observed both in BDL-rat livers and cholestasis patients. Furthermore, the expression of PGC1α, major inductor of mitochondrial biogenesis and key modulator of the expression of nuclear DNA-encoded respiratory enzymes and proteins involved in the transcription of mtDNA like mitochondrial transcription factor A (Tfam), falls during cholestasis[338]. Altogether, these data highlight the important role of mitochondria in cholestatic liver injury.

b) Hepatocyte death

BAs accumulation within the hepatocytes may result in cell injury and death via two mechanisms: whereas lower concentrations of BAs promote apoptosis, higher concentrations promote necrosis[335]. Both mechanisms have been observed in several cholestatic models and have been reported to play a role in cholestatic liver injury, nonetheless the contribution of each mechanism remains controversial. The major role of apoptosis in cholestatic liver injury has been demonstrated, although necrosis has been found predominant in some mouse models[6]. Mitochondrial membrane depolarization is a common event in BAs-induced hepatocyte apoptosis and necrosis. Mitochondrial membrane depolarization and the opening of the **MMTP** implicate the loss of the MMP, reduced OXPHOS, rupture of the outer mitochondrial membrane and importantly, the release of pro-apoptotic proteins into cytosol[337]. As previously commented, necrotic cell death is characterized by plasma membrane disruption, cellular and mitochondrial swelling, ATP depletion and the release of intracellular content. In the case of BAs, it may be induced by direct membrane damage by the detergent-like properties of BAs. In human cholestatic livers, features of hepatocellular necrosis, like massive hepatocyte swelling and increased serum aminotransferase enzymes have been observed[339].

On the other hand, it is well known that BAs may induce apoptosis in hepatocytes by activating the death receptors and the classic mitochondrial pathways[6]. Interestingly, BAs hydrophobicity does not always correlate with their cytotoxicity at the apoptosis level. BAs-induced hepatocyte apoptosis occurs through the death receptors **Fas** and **DR5**[340,341]. BAs stimulate Fas and DR5 expression and oligomerization independently of their ligands, leading to the initiation of a protease cascade. These events have been associated with BAs-induced oxidative stress considering that ROS can activate JNK and protein kinase C (PKC) and these at the same time may activate EGFR, which promotes the phosphorylation, translocation and activation of Fas[160]. Notably, increased Fas and DR5 expression also sensitizes hepatocytes to Fas and DR5-ligand-dependent apoptosis. Hence, toxic BAs boost death receptors-dependent hepatocyte apoptosis. After death receptors activation and DISC formation, activated procaspase-8/10 cleaves Bid to *t*Bid and leads to mitochondrial permeabilization, release of Cyt *c* and other pro-apoptotic proteins into cytosol and ultimately, apoptosis (intrinsic pathway)[6,160]. Even though DR5 has been suggested to be involved in BAs-induced apoptosis, the main death receptor mediating hepatocyte death is Fas. Another mechanism by which apoptosis occurs beyond BA-mediated injury includes the overexpression of TNFα and its interlinking with receptors TNFR1 and TNFR2 to activate the caspase pathway, as observed in BDL mice[342].

It has been recently demonstrated that hydrophobic BAs can also induce apoptosis by other cell death intracellular mechanisms, like **ER stress**[343]. Bile salts promote the expression of C/EBP homologous protein (CHOP), which is a sensitive marker of ER stress and strong activator of the ER stress-induced apoptotic pathway. High levels of CHOP lead to apoptosis by inducing the expression of the pro-apoptotic protein Bim and the death receptor DR5[6].

2.7.3.3 Main signal transduction pathways involved in cholestasis

BAs are not just digestive detergents but also major cell signaling molecules regulating important biological processes[331]. For this reason, hepatobiliary disorders like PBC, BAT and ALSaccount for the activation of many signaling pathways associated with BA metabolism, oxidative stress and cell death.

FXR (named nuclear bile receptor) plays a pivotal role in BA homeostasis regulating the expression of a battery of genes involved in BA synthesis, secretion, absorption and uptake[344] (Figure 2.17). Importantly, FXR prevents BAs accumulation by inhibiting their biosynthesis through the upregulation of **SHP** (inhibitor of the BA synthesis rate-limiting enzyme **CYP7A1**) and via the transcriptional induction of BA efflux transporters including BSEP, OSTα/β (organic solute transporter alpha and beta), MDR2 (multidrug resistance 2) and MRP2 (multidrug resistance-associated protein 2). The blockage of BA synthesis (CYP7A1 mRNA is significantly repressed in PBC, BAT and ALS livers) and the induction of basolateral BA export represent adaptive mechanisms to reduce BA burden in chronic cholestasis[345–347]. However, these adaptations do not counteract cholestatic liver injury. Besides FXR, there are other nuclear receptors like PXR (pregnane X Receptor), VDR (vitamin D receptor) and PPARα/γ that participate in BA metabolism and are considered therapeutic targets in obstructive cholestasis[348].

BAs have been reported to activate, besides nuclear receptors, MAPKs and stress activated protein kinases (SAPKs). It has been shown that BAs increase cAMP and induce (directly or by superoxide anions) the activation of ERK, p38 and PI3K/Akt pathways in murine and human hepatocytes, as an anti-apoptotic and survival response[334]. On the other hand, JNK activation by oxidative stress and pro-inflammatory cytokines like TNFα mediates the apoptotic response induced by BAs[335]. However, *in vitro* experiments using primary rat hepatocytes demonstrated that toxic BAs could also activate the JNK/c-Jun pathway to downregulate CYP7A1 and inhibit BA synthesis, thus protecting hepatocytes against toxicity[349]. As mentioned above, PKC may also be activated by BAs and participate in BA hepatotoxicity via Fas translocation and activation. Moreover, as has been observed in primary hepatocytes treated with DCA, BAs can activate EGFR too[350] and stimulate the downstream Raf-1/MEK/ERK signaling cascade[351].

Finally but equally important is the activation of the transcription factor **NFκB** in hepatocytes during

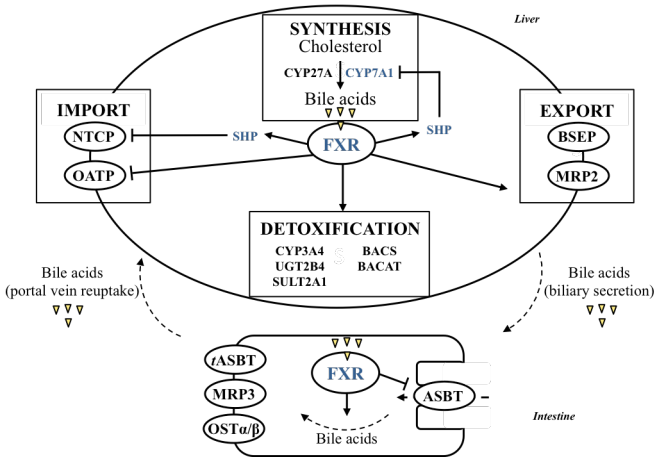

Figure 2.17 Central role of FXR regulating bile acid metabolism and circulation between the liver and the intestine. FXR negatively regulates BAs production by repressing CYP7A1 the rate-limiting enzyme of the synthetic pathway. FXR also induces the expression of BACS and BACAT, enzymes involved in BA conjugation, activates the expression of the BA export transporters MRP2 and BSEP and simultaneously represses BA import by downregulating NTCP and OATP. Furthermore, FXR induces the expression of the CYP3A4, UGT2B4, and SULT2A1 enzymes involved in the detoxification of BAs. Subsequently, sulfated/glucuronidated BAs are exported by MRP2 into the canaliculus. At the intestinal level, FXR influences the import of BAs by interfering with the transcription factor network controlling ASBT. Adapted from[344].

cholestasis. The nuclear localization of NFκB both in livers from BDL-mice and in primary hepatocytes treated with toxic BAs confirms its activation[352]. Whereas in resting hepatocytes NFκB localizes in the cytosol bound to the molecule IκB in an inactive form, it is activated during cholestasis. BAs stimulate IκB phosphorylation and proteasomal degradation with the subsequent translocation of NFκB (as a p65/p50 complex) into the nucleus where it binds to DNA and induces the expression of specific target genes involved in cell survival such as X-linked inhibitor of apoptosis protein (XIAP)[353]. Furthermore, NFκB inhibition in BDL-mice increased around three-folds the hepatic damage, corroborating its major protective role reducing liver injury during obstructive cholestasis.

2.7.3.4 Inflammatory and fibrotic responses

The different stages of cholestatic liver diseases are defined by the degree of bile duct damage, inflammation and fibrosis[43,354]. As it occurs in most chronic liver diseases, hepatocyte injury all along during cholestasis is responsible for the later inflammatory and fibrogenic responses of non-parenchymal cells. It is well-known that damaged hepatocytes release a variety of molecules, including cytokines, growth factors and lipid peroxide products that may exacerbate the inflammatory response, promote fibrogenesis and injure other adjacent cells.

Given the fact that BAs can activate signaling pathways that stimulate the production of different mediators of inflammation, they are considered inflammatory agents[332]. According with this, it has been shown in the murine model of BDL that neutrophils and macrophages are activated and recruited into the liver during obstructive cholestasis, causing significant liver damage[355,356]. This inflammatory response correlated with increased levels of pro-inflammatory cytokines like **TNFα**, **IL-6** and IL-1β, chemokines such as CCL2 and CXCL1 (C-X-C motif chemokine ligand 1), enhanced expression of the adhesion molecules ICAM1 (intercellular adhesion molecule 1) and VCAM1 (vascular cell adhesion molecule 1) as well as other proteins that impact the immune response like plasminogen activator inhibitor (PAI1).[357,358] Interestingly, mice and human studies have demonstrated that the up-regulation of hepatic EGR1 (early growth response 1) by BAs during cholestasis correlates with the expression of some of these genes suggesting an EGR1-dependent inflammatory regulation in cholestatic liver injury. As commented in the previous section, NFκB plays a major role protecting hepatocytes against BA toxicity and its activation in cholestasis has also been linked to liver cancer development[359]. Finally, even though the role of KCs in cholestasis remains controversial, most studies present evidences of KCs activation and cytokine production upon interaction with BAs[360]. Overall, these observations demonstrate the strong implication of inflammation in liver injury associated to obstructive cholestasis.

Along with inflammation, hepatic and biliary fibrosis can develop as cholestasis progresses. Actually, there are many factors that can cause HSC activation and hepatic **ECM** deposition during cholestasis. For instance, both nuclear receptors and BAs themselves can induce the transdifferentiation of HSCs to an activated state and promote the synthesis of collagen and other ECM proteins. Notably, BAs bind to EGFR of HSCs activating downstream proliferative signaling pathways[348,361]. Moreover, it has been shown that BAs stimulate the expression of TGFβ in hepatocytes and KCs, which strongly contributes to fibrogenesis[332]. At the end, constant injury to small bile ducts may trigger BECs proliferation and fibrosis (caused by adjacent activated HSCs) and lead to biliary cirrhosis. Similarly, the continuous hepatocyte injury and the concomitant inflammation, proliferation and scarring of the liver may lead to hepatic cirrhosis and loss of liver function. In rare cases, increased nodular regeneration throughout the liver can progress to HCC[362].

2.7.3.5 BDL mouse model of obstructive cholestasis

BDL is the most common used experimental model of obstructive cholestasis in research[355]. The surgical ligation of the common bile duct in rodents leads to a cascade of pathological processes that result in cholestasis, inflammation and a strong fibrotic response. This model serves to examine the cellular and molecular mechanisms underlying these pathological events caused by incorrect bile flow. Among all the different surgical techniques for BDL, the complete obstruction of the

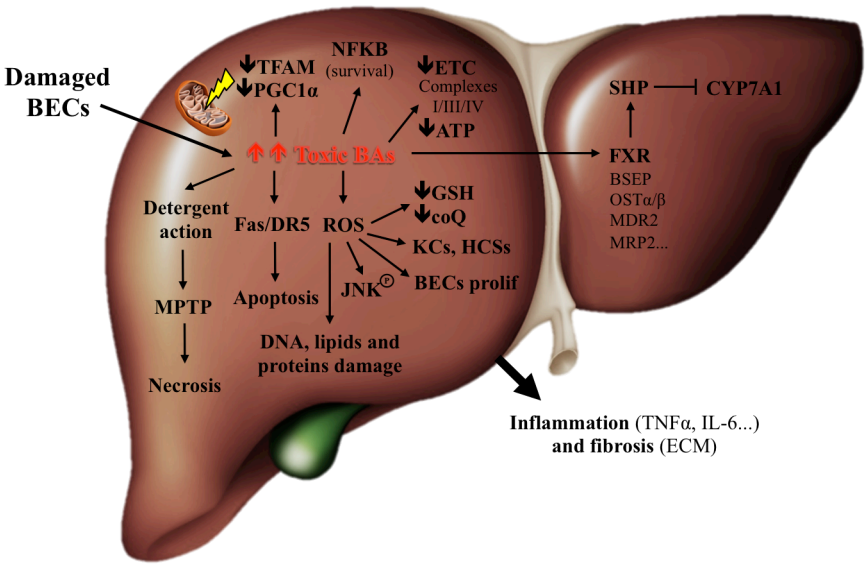

Figure 2.18 Main pathways implicated in the pathogenesis of obstructive cholestasis. The accumulation of toxic BAs within the liver causes a series of processes that lead to hepatocyte death, inflammation and scarring of the liver. BAs are hydrophobic salts that induce necrosis (MPTP opening), apoptosis (Fas/DR5 expression and oligomerization), oxidative damage (ROS are produced and GSH and coQ depleted) and mitochondrial dysfunction along with decreased mitochondrial biogenesis. Hepatocyte injury and BAs theirselves stimulate the release of pro-inflammatory cytokines and chemokines and the activation of HSCs (ECM production). As survival mechanisms BAs promote NFκB nuclear translocation and FXR activation.

common bile duct is the most aggressive and used. This technique is easily reproducible and induces a strong fibrotic respone in 21 days, but also results in high mortality rates due to infectious complications. Remarkably, the morphological changes observed in BDL are comparable to those in human biliary cirrhosis[363]. Rapidly after the ligation of the common bile, BAs accumulate within the liver producing significant hepatocellular necrosis and inflammation (leukocytes/macrophages infiltration) as seen by histological analysis. Like in humans, this inflammatory response leads to HSC activation and ECM deposition which may further compromise tissue structure and integrity and ultimately, liver function. The values of serum transaminases (both AST and ALT) are good markers for BDL-induced liver injury[355].

2.7.4. Major pathways implicated in the pathogenesis of HCC

As previously discussed in section 3.1.1.4, HCC is the main primary liver cancer, the fifth most common cancer worldwide and the second leading cause of cancer-related deaths[69]. This is in part because HCC is a poor prognosis cancer as it does not present symptoms in its early stages and in more than half of the cases it is detected at an advanced stage[70–72]. Even though treatment options have improved in the past few years, there is still no standard procedure for its treatment and therapies remain palliative. For all these reasons, finding new therapeutic tools and approaches to manage HCC is crucial.

HCC is considered phenotypically and genetically a very heterogeneous cancer whose pathogenesis is characterized by a **complex network of signaling pathways**[74–77]. HCC is thought to be caused mainly by two mechanisms: chronic liver injury associated to multiple etiologies (viral infections, alcohol, drugs, toxins, metabolic and autoimmune disorders etc) or genetic alterations (mutations or changes in the expression of cancer-related genes such as oncogenes and tumor suppressors). Both mechanisms have been associated with alterations in important signaling pathways involved in hepatocarcinogenesis. On this matter, the main signaling pathways associated with hepatic malignant transformation include p53, Rb, HIF-1α, c-Myc, Wnt/β-catenin, Ras/Raf/MEK/ERK, PI3K/Akt/mammalian target of rapamycin (mTOR) NFκB and JAK/STAT (janus kinase/signal transducer and activator of transcription) pathways and growth and angiogenic factors such as HGF (hepatocyte growth factor), IGF (insulin-like growth factor), PDGF (platelet-derived growth factor) and VEGF, among others[364,365]. In the last few years, several studies have demonstrated oncogenic properties of other proteins in HCC, like Liver kinase 1 (LKB1)[366] and HuR[367,368], whose altered expression and signaling may lead to metabolic reprogramming, aberrant cell proliferation and apoptosis resistance.

In here, a brief description of some of the main molecular mechanisms implicated in the development and the progression of HCC will be provided (Figure 2.19).

2.7.4.1 Metabolic reprogramming: The Warburg effect

Otto Warburg postulated in the 1920's that cancer cells undergo a metabolic reprogramming in order

to proliferate in a hostile environment[369]. He described how tumor cells produce energy mainly in the cytosol, independently of oxygen, by high rates of **"aerobic glycolysis"**. Under this situation, despite the presence of oxygen, glycolysis is followed by lactic fermentation instead of OXPHOS in mitochondria. Indeed, tumor cells present typically glucose consumption rates 200 higher than their precursor cells[370]. The Warbug effect allows cancer cells to use glucose and its intermediate metabolites to produce reducing equivalents like NADPH, nucleotides, proteins and lipids that are required for cell division and apoptosis suppression[371]. Increased aerobic glycolysis is a common feature in most human cancers, including HCC, and very often correlates with tumor aggressiveness and poor prognosis[372]. However, the molecular mechanisms that link this metabolic change with cell proliferation and apoptosis resistance are not fully understood.

Several studies have linked Ras, PI3K/Akt/mTOR, LKB1/AMPK/mTOR and HIF-1α pathways with glycolysis, cancer metabolism and cell proliferation, key processes for the Warbug effect. In the same way, the loss of tumor suppressors like p53 and the activation of oncogenes like c-Myc have been associated with alterations of pro-survival signaling pathways that sustain the Warburg effect[371]. Importantly, all these pathways play major roles in liver cancer initiation and progression, which is in accordance with the close association between metabolic alterations and HCC pathogenesis. Moreover, experiments where liver cancer cells metabolism was interfered resulted in decreased carcinogenicity and increased apoptotic response to chemotherapeutic agents, reinforcing the central role of aerobic glycolysis in tumor growth and survival[373,374].

Even thought it has been always thought that tumor cells have reduced OXPHOS due to low oxygen availability and decreased mitochondrial respiration and TCA cycle activity, some recent investigations have observed intact OXPHOS function in most cancers, suggesting that the relationship between glycolysis and OXPHOS may be cooperative in carcinogenesis[375].

2.7.4.2 PI3K/Akt/mTOR pathway

The **PI3K/Akt/mTOR** signaling pathway is one the main survival pathways in the cell and is found **abnormally activated in HCC**[376]. The PI3K/Akt pathway, which is the main mediator between the cell and extracellular signaling, is activated by growth factor receptors like EGFR and IGF receptor (IGFR). These receptors stimulate the transmission of mitogenic signals to mTOR, which ultimately coordinates the cellular energy/nutrient status with cell growth and proliferation by activating S6 kinase, enzyme that regulates protein synthesis and the transition from G1 to S phase of the cell cycle[377]. It has been observed that mTOR phosphorylation; the expression of its downstream effector S6K1 and the activity of S6 are increased in approximately 50% of HCCs[77,378]. Furthermore, the activation of the PI3K/Akt/mTOR pathway has been correlated with advanced and poor prognosis HCCs. Immunohistochemistry (IHC) analysis have demonstrated that Akt and S6 phosphorylation associate with poor survival of HCC patients[378]. All these results point out the major contribution of the PI3K/Akt/mTOR pathway to HCC progression. However, to date, the mechanisms underlying this common and aberrant activation remain poorly understood.

Most HCCs with Akt/mTOR activation do not present genetic alterations, suggesting an indirect activation by growth factor receptors. In fact, the activation of upstream receptors is considered one key mechanism in this pathway and include the overexpression of c-Met, EGFR and IGFR, which are all significantly increased in human HCC and have shown oncogenic properties in HCC mouse models[379]. On the other hand, reduced expression of phosphatase and tensin homolog (PTEN), inhibitor of Akt, is found in nearly half of all HCC tumors resulting in constitutive activation of the PI3K/Akt/mTOR pathway. Although *PTEN* gene mutation rarely occurs (5%), loss of heterozygosity of *PTEN* allele has been identified in approximately 30% of HCC patients[77,380]. Other mechanism of PI3K/Akt/mTOR pathway overactivation in cancer is the mutation or amplification of the *Akt* gene (mainly *Akt1* and *Akt2* isoforms)[381]. Akt2 elevated expression, but not Akt1, has been reported in 40% human HCCs correlating with prognosis[382]. Finally, somatic mutations in the PI3K catalytic α gene *PIK3CA* have also been reported in HCC[383].

Akt is hyperactivated in HCC and has been associated with liver carcinogenesis through the regulation of a variety of processes including cell survival by enhancing Mdm2-mediated ubiquitination and degradation of p53; cell cycle by activating and stabilizing cyclin D1; cell growth by activating mTOR; and metabolism by regulating both the Warburg effect and OXPHOS[381,384,385]. Noteworthy, **Akt is considered a "Warburg kinase"**[386]. Besides HCC, deregulation of the PI3K/Akt/mTOR pathway in the liver is a common molecular event that has been associated with altered metabolism and fibrosis/cirrhosis development, as observed for example in human NAFLD, hepatitis virus infections and the BDL mouse model of obstructive cholestasis[387–390]. These evidences along with the beneficial effects of targeting this pathway in HCC patients[376], indicate the relevant role of the PI3K/Akt/mTOR pathway in liver cancer.

2.7.4.3 LKB1

Liver kinase B1 (LKB1) is an ubiquitously expressed serine/threonine protein kinase that has a major role in metabolism. It was identified originally as the tumor suppressor gene responsible for the inherited cancer disorder Peutz-Jeghers Syndrome but has been identified as a tumor suppressor in many other cancers. Indeed, somatic mutations in *STK11* (gene that encodes LKB1) have been described in several cancer types including colorectal, pancreatic, breast and lung cancers[391,392]. Interestingly, LKB1 expression can also be regulated by epigenetic and PTMs. Even though KO

mice studies have shown that *STK11* deletion of both alleles is embryonic lethal and hence, LKB1 function critical for development, heterozygous LKB1$^{+/-}$ mice develop polyps in the gastrointestinal tract and HCC[393]. In this line, mice bearing a liver-specific *STK11* deletion had increased expression of SREBP-1 target genes, and hepatic lipid accumulation and steatosis.

Whereas LKB1 is mostly nuclear in mice, in humans it is localized both in the nucleus and cytoplasm. In mammals, LKB1 forms a trimeric complex with STRAD (STE-20-related adaptor) and MO25 (mouse protein 25) that stimulates its cytoplasmic localization and kinase activity[394]. Importantly, it has been shown that LKB1 cytosolic localization is critical for its role as tumor suppressor, since LKB1 mutants are not able to translocate into the nucleus and inhibit cell growth[391]. Regarding cell energy metabolism, **LKB1 is the major upstream kinase of AMPK**. Under energy stress, LKB1 phosphorylates and activates AMPK, key cellular energy and metabolic sensor, resulting in the stimulation of energy producing processes and the inhibition of the energy demanding ones in order to restore and maintain cellular homeostasis. In this process AMPK negatively regulates mTOR signaling. Along with AMPK, there exist around 12 other AMPK-related kinases that can be phosphorylated and activated by LKB1. Besides its functions as tumor suppressor and metabolic regulator, LKB1 is also known to regulate cell growth, apoptosis and cell polarity[392]. It has been described that its functions regulating cell growth and apoptosis are linked to the tumor suppressor p53, with which it interacts directly. Moreover, recent findings have shown new features of LKB1 regulating cell survival; like that it is essential for proliferation in cell lines with Akt constitutively active[395].

In the last few years it has been shown that **LKB1 has an oncogenic role in liver cancer.** Studies with MAT1A-KO mice that develop spontaneously NASH and HCC revealed that the LKB1/Akt pathway plays a critical role in the proliferation of HCC from NASH. In agreement with this, the SAMe-D cell line which was isolated from MAT1A-KO HCCs and is characterized by low SAMe, presents increased cytoplasmic levels of phosphorylated LKB1 regulating cell survival through direct interaction and activation of Akt, independently of PI3K, AMPK or mTOR[366]. Likewise, it was observed in GNMT (Glycine N-methyltransferase)-KO mice, which also develop spontaneously NASH and HCC[396], that LKB1 was hyperactivated by Ras, one of the main oncogenes involved in the initiation and progression of liver cancer, contributing to cell proliferation and HCC development. Confirming this, studies using the OKER cell line, which was isolated from GNMT-KO HCCs and is characterized by high SAMe, showed that increased intracellular levels of SAMe promote epigenetic regulation of the Ras and JAK/STATs inhibitors, RASSF1/4 (Ras association domain-containing protein 1/4) and SOCS1/3 (suppressor of cytokine signaling 1/3), leading to the aberrant hyperactivation of the Ras signaling pathway with subsequent increased cAMP levels, hyperphosphorylation of cytoplasmic LKB1 and hypophosphorylation of AMPK. Moreover, *in vivo* experiments demonstrated that LKB1 silencing induces apoptosis and HCC regression due to the inhibition of Ras activity[397]. Finally and importantly, LKB1 overactivation has been associated with poor outcomes in HCC patients[397]. Overall, these data evidence the importance of LKB1, until now considered a tumor suppressor, in cell proliferation and survival in liver tumors derived from metabolic disorders, providing a new vision for the role of LKB1 in tumorigenesis[392].

2.7.4.4 HuR

HuR is an ubiquitously expressed protein belonging to the Elav/Hu family of RNA-binding proteins that plays major biological roles regulating gene expression by binding to and stabilizing mRNAs containing AU-rich elements[367]. Upon stimulation, HuR translocates into cytosol where it stabilizes target mRNAs encoding important genes for cell cycle control and proliferation like cyclin A2, B1, D1 and E1, thus promoting cell growth and survival. Notably, among HuR targets there also exist proto-oncogenes like c-Myc and c-Fos. On the other hand, under situations like genotoxic stress, HuR may exert an anti-proliferative role by targeting pro-apoptotic p53 and cell cycle inhibitor p21 mRNAs, interfering cell growth[398,399]. In spite of this, the role HuR has been primarily associated to proliferation along with angiogenesis, invasiveness and metastasis, supporting its role in tumorigenesis. Indeed, HuR expression is frequently increased in colon, breast, prostate and many other cancers, correlating with tumor malignancy[399,400].

The role of HuR in liver carcinogenesis has also been described[367,368]. HuR is known to regulate hepatocyte dedifferentiation, proliferation and survival, three major hallmarks of cancer. Moreover, HuR increased expression along with a significant higher cytoplamic localization have been reported in HCC patients with a variety of etiologies[368]. Similarly, high cytoplasmic HuR has been reported in cirrhotic livers[401,402]. Worth noting that HuR activity is regulated by LKB1 in SAMe-D cells isolated from MAT1A-KO HCCs[366]. Altogether, these findings support the involvement of HuR in liver malignant transformation.

Regarding HuR regulation, it is well known that NF-κB can activate HuR transcription downstream of the PI3K/Akt signaling pathway[403]. On the other hand, it has been shown that in response to moderate heat shock, the levels of HuR transiently and potently decrease. This abundance reduction is linked to the ubiquitination and proteasome-mediated degradation of HuR protein that, in fact, enhances cell survival to the heat shock stimulus[404].

Finally, in 2012 the mechanism underlying HuR overexpression in HCC and colon cancer was finally unraveled. In this study it was shown that HuR levels correlate with the expression of the oncogene Mdm2, both in human HCC and colon cancer metastases. In addition, it was observed that Mdm2 stabilizes HuR

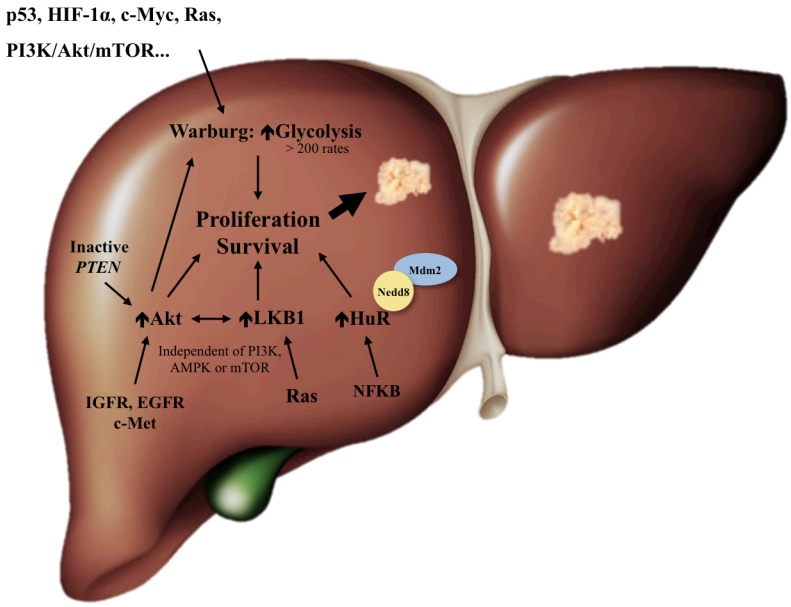

Figure 2.19 Major pathways implicated in the pathogenesis of HCC. HCC is a ver complex disease with multiple signaling pathways converging in the same malignant transformation. Liver injury caused by different factors such as steatosis, cholestasis, viral infections or toxins stimulate the activation of proliferative and survival signaling pathways that lead to aberrant cell growth and carcinogenesis. Some of these pathways are associated with changes in mitochondrial metabolism and the Warburg effect, which give cells proliferative and survival advantages (tumoral features). Along with Akt, which is strongly involved in the development of a variety of cancers, LKB1 and HuR are highly induced in liver cancer. In the last few years it has been discovered that LKB1, despite its tumor suppressor role in other tissues, is an oncogenic driver in HCC. Moreover, it has been shown that HuR is stabilized and overexpressed in HCC by Mdm2-mediated neddylation.

through neddylation promoting its nuclear localization and protection from degradation. These data suggest the novel Mdm2/Nedd8/HuR regulatory framework essential for the malignant transformation of tumor cells[367].

2.8 THERAPEUTIC APPROACHES FOR LIVER DISEASE

The treatment options recommended for DILI, NAFLD, cholestatic liver diseases and HCC depend, among other factors, on the extent of the disease and the liver function, being liver transplantation, in all cases, the last resort for cure. This section will give an overview of the actual treatments for each disease.

- **DILI** is a leading cause of ALF and transplantation in the US and most of Europe and in few cases it may progress to chronic liver disease[21,22,28]. The main cause of DILI is the abusive intake of drugs (primarily APAP), but there are evidences supporting the role of other factors such as sex, age and chronic liver disease in its development[405,406]. An early recognition of drug-induced liver reactions is essential to minimize injury. In case of fulminant hepatic injury, transplantation appears to be the only life-saving procedure[407].

There is no specific treatment for DILI. The first step to take is to discontinue the suspected drug and the therapy is limited to the use of *N*–acetylcysteine (NAC), precursor of GSH, in the initial phases of hepatotoxicity[408,409]. In cases of valproate toxicity, L-carnitine is potentially valuable[410].

- **NAFLD**, as already mentioned is the most common chronic liver disease in Western world and is becoming a major health problem worlwide[31,32]. It is associated to obesity, type 2 diabetes and metabolic syndrome, disorders with increasing prevalence in the general population[13,33]. Although steatosis rarely progresses to advanced diseases, around 20% of NASH patients develop fibrosis and ultimately cirrhosis and are at risk of developing liver-related complications such as portal hypertension, hepatic failure and HCC[37–39]. It should be noted that NAFLD is the third most common cause for liver transplantation in the United States[40].

Nowadays no specific medical treatment for NAFLD exists and lifestyle changes like an adequate diet and exercise are the main basis of therapy. Indeed, it has been shown that 7-10% of weight loss slows the progression of NAFLD and may reverse some of the steatosis and neroinflammation[411]. There are few clinical trials in NAFLD and no specific therapy can be firmly recommended. For this reason, drug therapy should be reserved for patients with progressive NASH or with high risk of disease progression (diabetes, metabolic syndrome, persistently increased ALT or high necroinflammation) to prevent disease progression[34,412].

Since NAFLD is strongly associated to IR and metabolic syndrome, its management with oral antidiabetics, lipid-lowering agents and antihypertensive

drugs is suggested for patients with associated type 2 diabetes, dyslipidemia and/or arterial hypertension, respectively. Additionally, drugs like Pioglitazone (antidiabetic) and Losartan (antihypertensive) have demonstrated beneficial effects and hepatic histologic improvements. Another agent that has shown good efficacy improving histological liver damage is vitamin E, which is an antioxidant that should be administrated only to non-diabetic and histologically confirmed NASH patients, as it has adverse effects. Insulin-sensitizing molecules with anti-inflammatory and fibrotic properties are now under investigation: FXR agonists like obeticholic acid and PPAR α/δ agonists[413,414].

- **Cholestatic liver diseases**, as described before, are a very heterogeneous group of diseases. Depending on the type and degree of the disease, the cholestatic condition can be progressive and lead to biliary cirrhosis or more severe and require urgent liver transplantation[44,45].

Although survival of PBC patients has been largely improved with the use of ursodeoxycholic acid (UDCA), one third of patients still do not respond to the treatment and require liver transplantation[56]. The main goals of cholestatic liver diseases' treatments are to alleviate symptoms such as fatigue, pruritus and osteoporosis, correct fat-soluble vitamin deficiencies and slow the progression of the disease. Regarding BAT and other childhood cholestatic liver diseases, the only effective treatment is early surgery. Given that after liver transplantation the majority of patients does not restore bile flow and develop secondary biliary cirrhosis, the best option is to restore bile flow by Kasai procedure within the first 2 months of life. It should be noted that Kasai procedure not always restores bile flow and patients may develop cirrhosis and other complications requiring liver transplantation[59]. UDCA is being administrated to infants with BAT after operation to improve jaundice and bile secretion but one third of patients do not respond to the treatment and die of hepatic failure[415]. Long-term BAT survivors with native liver are infrequent. For ALS, the standard treatment is medical and based on alleviate symtoms like pruritus and fat-soluble vitamin deficiencies. Biliary diversion may be very helpful and can be useful before liver transplantation. Progressive liver disease, eventually causing cirrhosis and failure, and requiring liver transplantation occurs in approximately 15% of cases[61]. As already commented ALS is a poor prognosis disease with a survival rate of 20-years in 80% of patients that do not require liver transplantation and 60% in those that do require transplantation[63].

In general, there are no standard procedures for cholestatic liver diseases and specific surgery and UDCA treatment are the only available but not completely satisfactory treatments.

UDCA, which increases bile flow and reduces the toxicity of more hydrophobic BAs through its membrane-stabilizing effect, is the most used pharmacological agent for cholestatic liver diseases and has shown significant clinical, biochemical, and histologic improvements in patients with early stages of PBC and also delays the average time before transplantation is necessary. However, the efficacy of this medication in advances stages like cirrhosis is questionable. UDCA is known to inhibit hepatocyte apoptosis by regulating the opening of the MMTP, Bax translocation, Cyt *c* release and the subsequent caspases activation[416]. As mentioned above, some patients do not respond to UDCA treatment and require alternative or additional therapies.

Immunosuppressive agents such as corticosterioids, methotrexate and cyclosporine A are other treatments that have shown biochemical and histological improvements in various trials but are potentially harmful during long-term administration and cannot be recommended for standard treatment[417]. Tauroursodeoxycholic acid (TUDCA) administration has also demonstrated therapeutic effects against cholestasis, but long term clinical trials are required to fully evaluate its effectiveness[418]. Finally, FXR agonists have shown improved serum alkaline phosphatase levels and other hepatic biochemicals in cholestasis patients. Currently, nuclear receptors RXR, PXR and PPARα, membrane receptors fibroblast growth factor receptor 4 (FGFR4) and apical sodium BA transporter (ASBT), and the agent *nor*UDCA, a 23-C homologue of UDCA, are under investigation as promising targets for the treatment of cholestatic disorders[419].

- **HCC** is an extremely complex and heterogeneous disease with many different signaling pathways converging in the same malignant transformation[74–77]. Moreover, HCC is considered a poor prognosis cancer since it does not present symptoms in its early stages and in more than 50% of the cases is detected at an advanced stage, presenting multiple HCCs and underlying cirrhosis[70–72]. Even though treatment options have improved in the past few years, there is still no standard procedure for treating advanced HCC, due to the wide variety of liver diseases associated to its pathogenesis, and therapies remain palliative. Furthermore, HCC treatment options also depend on the comorbidities present in the patient. The median survival following diagnosis is approximately 6 to 20 months but when the cancer is not completely removed, the disease is usually fatal[73].

HCC management is based on the Barcelona clinic liver cancer (BCLC) allocation system[420], and can be divided into surgical and non-surgical therapies:

Surgical therapies are potential curative approaches for HCC and include hepatic resection, which consists of removal of tumor and surrounding liver tissue while preserving enough liver for normal function and liver transplantation, that remains the best option but is very limited due to the scarcity of donors[421,422]. Hepatic resection is recommended for patients with solitary tumors and very well-preserved liver function and not for very advanced HCCs with multiple nodules and surrounding cirrhotic liver. These treatments have shown

good prognosis and long-term survival, but unfortunately only around 15% of HCCs can be completely removed. Liver transplantation, on the other hand, should be considered in any HCC patient with cirrhosis and a small single nodule of 5 cm or less or up to 3 nodules of 3 cm or less. The survival rate after transplantation has increased over the years but this approach is curative for patients with advanced HCC and no extrahepatic metastasis.

Alternatively, when surgery is not possible, **non-surgical therapies** can be used to prevent HCC progression and delay recurrence[70,423]. These therapies include: i) local ablation with radiofrequency (RFA) and percutaneous ethanol injection (PEI), techniques that destroy abnormal tissue using high frequency ultrasound or ethanol injections respectively and are considered the standard of care for patients at early stages of HCC not suitable for surgical therapies; ii) transcatheter arterial chemoembolization (TACE), which is the most common offered therapy for unresectable HCCs with preserved liver function and absence of vascular invasion or extra hepatic spread, consisting of the injection of a cytotoxic agent (like doxorubicin) into the hepatic artery, thus killing cancer cells and iii) radioembolization, which consists of the intrahepatic application of radioactive substances such as Iodine-131-labeled lipiodol or microspheres containing Yttrium-90 via the hepatic artery to destroy diffuse or multifocal liver tumors and have shown increased survival rates in low-risk patients.

And finally, the **molecular therapy**, which include therapeutic agents that target different signaling pathways implicated in HCC pathogenesis[424]. Sorafenib, which is the standard systemic therapy for HCC, is a multikinase inhibitor of the Raf/MAPK/ERK signaling pathway that inhibits both tumor growth and angiogenesis (it blocks VEGF, PDGF and c-Kit receptors) and has shown improvements in survival (around 3 months) in phase III clinical trials for advanced HCC. Sorafenib represents a big advance and the most promising chemotherapeutic agent in the treatment of inoperable HCCs with preserved liver function. However, there also exist side effects and loss of efficacy over the time. Everolimus is an inhibitor of mTOR that has also shown anti-tumoral properties against HCC in xenografts and is now being studied in phase II trials in metastatic disease. Interestingly, the combination of both agents has shown promising early results. At this time, similar agents are under investigation.

These treatments have not shown the greatest efficacy and there are many other agents like doxorubicin, tamoxifen and antiadrogens, which have been studied and tried with minimal success[425]. Currently, liver-directed therapies such as resection, transplantation and RFA are the only offering hope for extended survival in patients with advanced HCC. Future research may include systemic chemotherapy, newer targeted agents, and immune therapy.

3. HYPOTHESIS AND OBJECTIVES

3. HYPOTHESIS AND OBJECTIVES

The liver is the largest and most metabolically active solid organ in the human body. It participates in vital functions like digestion, detoxification and metabolism, being essential for energy and nutrient distribution to the entire body and also for blood clearance of toxic substances. Due to its central role in metabolism and clearance it is exposed to many harmful agents, which makes it more susceptible to injury than other organs. This injury can be chronic and evolve with time to cirrhosis and cancer, or be acute and lead to fulminant liver failure. There are more than 100 known causes of liver injury, although it is stereotypically linked to alcohol and viral infections. In the last few years, bad dietary habits along with obesity and type 2 diabetes and excessive drug consumption have become emerging risks factors for developing liver disease. It should be noted that genetic mutations and autoimmune disorders are other major causes of liver disease that account for thousands of deaths annually. In many cases, liver injury is detected at an advance stage and the treatment options for many diseases are clearly unsatisfactory. Hence, the incidence and mortality of liver disease is increasing dramatically all over the world and currently represent a global major health problem. Unraveling new mechanisms underlying these malignancies that pave the way for the development of new tools for its early diagnosis and treatment is crucial.

Mitochondria play a central role in cellular energy homeostasis, metabolism and cell death. Due to the tremendous metabolic activity of the liver, it is one of the richest organs in number of mitochondria and one of the most susceptible to disorders that affect mitochondrial functions. Indeed, mitochondrial alterations have been documented in a variety of acute and chronic liver diseases and have been recently proposed as a common mechanism in their pathogenesis. It has been shown that defects in the mitochondrial respiratory chain trigger liver injury in DILI, NAFLD and cholestatic liver disease and moreover, that the energetic misbalance and ROS production due to mitochondrial dysfunction contributes largely to liver fibrogenesis and carcinogenesis. There are several mitochondrial proteins that have been reported to have a role in liver pathology, among them PHB1 and MCJ. These proteins have shown opposite effects in mitochondrial function and two knockout mouse models in which PHB1 and MCJ genes are inactivated have been developed: i) mice lacking *MCJ* gene (MCJ KO mice) represent a model of increased mitochondrial function and have shown accelerated hepatic lipid metabolism under altered metabolic conditions and ii) mice lacking *PHB1* gene (*Phb1* KO mice) represent a model of decreased mitochondrial function and spontaneously develop liver fibrosis, bile duct metaplasia and HCC. These animals represent good models to study the relationship between altered mitochondrial function and liver pathogenesis.

On the other hand, post-translational modifications are considered key mechanisms maintaining protein homeostasis and normal cellular signaling, metabolism and function in eukaryotic cells. In the liver, PTMs influence almost all aspects of normal cell biology and aberrant modifications have been associated to different hepatic disorders. Alterations in the acetylation, ubiquitination and neddylation pathways have been reported in a variety of human liver pathologies and seem to play a major role in the heterogeneity and versatility of diseases. Interestingly, the lack of PHB1 has been shown to induce regulatory changes in histone acetylation and a down-regulation of proteasome activity. On the other hand, it has been demonstrated that the oncogenic driver HuR is stabilized by neddylation in liver and colon cancer, suggesting the involvement of this PTM in liver carcinogenesis.

Therefore, a comprehensive characterization of the role of PHB1 and MCJ in liver injury associated to mitochondrial dysfunction and a better study of deregulated acetylation, ubiquitination and neddylation in liver malignancy will uncover new insights of this pathology and step forward for the development of new therapies.

OBJECTIVES

The principal aim of this study is to unravel new mechanisms underlying liver pathogenesis regarding mitochondrial dysfunction and aberrant protein homeostasis, in order to identify new possible therapeutic strategies to treat liver disease.

For that purpose we have established the following objectives:

1. Characterize the role of MCJ in liver physiology in terms of mitochondrial metabolism.

2. Examine the possible implication of MCJ in DILI pathogenesis and evaluate the impact of its deficiency, and presumably increased mitochondrial function, on drug-induced liver injury in the experimental mouse model of APAP hepatotoxicity as a therapeutic approach to treat human DILI.

3. Examine the possible implication of MCJ in NAFLD pathogenesis and evaluate the impact of its deficiency, and presumably increased mitochondrial function, on lipid metabolism and progression of the disease in an experimental mouse model of NASH as a therapeutic approach to treat human NAFLD.

4. Investigate the role of PHB1 in cholestatic liver disease at both mitochondrial and nuclear levels, focusing mainly on the acetylation and ubiquitination pathways.

5. Study the involvement of the neddylation pathway in liver carcinogenesis aiming to identify new Nedd8 targets in HCC and test the efficacy of targeting neddylation as a promising strategy to treat HCC.

4. EXPERIMENTAL PROCEDURES

4. EXPERIMENTAL PROCEDURES

4.1 HUMAN SAMPLES

All the studies were performed in agreement with the Declaration of Helsinki, and with local and national laws. The Human Ethics Committee of each hospital approved the study procedures and written informed consent was obtained from all patients before inclusion in the study.

4.1.1 Healthy controls

Healthy human liver (n=5-7) and serum (n=5-50) samples from Basque Biobank (Vizcaya, Spain) and Marqués de Valdecilla University Hospital (Santander, Spain) were used as controls. All had histologically normal liver, BMI<25 kg/m^2, normal fasting glucose, cholesterol and TGs, normal AST and ALT, and no evidence of hepatitis B and/or C viral infection as well as human immunodeficiency viral infection.

4.1.2 DILI

A total of 21 liver samples from patients with DILI were included in this study. The diagnosis of DILI, established in Virgen de la Victoria University Hospital (Málaga, Spain), was based on clinical data, features of liver histology and exclusion of other possible causes of liver injury (viral hepatitis, biliary diseases, alcohol abuse, NAFLD, autoimmune liver diseases, and hereditary diseases). DILI was clinically divided according to the type of liver injury (hepatocellular, cholestatic and mixed type), and their characteristics are summarized in Table 4.1. The causative agents were as follows: Amoxicillin/clavulanate, asparaginase, atorvastatin, azathioprine, bentazepam (2 cases), ciprofloxacin, cloxacilin, fluvastatin, fosfocreatine/taurine, cloxacillin, fluvastatin, fosfocreatine/taurine, gestodene, phenytoin, stanozolol, telmisartan, tetrabamate and tranilast.

4.1.3 NAFLD

Liver samples used come from 21 patients with a clinical diagnosis of NAFLD who underwent a liver biopsy with diagnostic purposes in Santa Cristina Hospital (Madrid, Spain), and serum samples come from 141 morbidly obese patients recruited through the Department of Digestive Surgery and Liver Transplantation (Nice hospital, France) where they underwent bariatric surgery for their morbid obesity. Inclusion criteria for NAFLD patients were based on an alcohol intake lesser than 20 g/day, the presence of biopsy-proven steatosis with/without necroinflammation and/or fibrosis, and no evidence of hepatitis B and/or C viral infection as well as human immunodeficiency viral infection. The characteristics of the study groups are described in Tables 4.2 and 4.3 respectively.

Clinical examination included a detailed interview with special emphasis on both alcohol intake and medications use, history of known diabetes and arterial hypertension, as well as measurements of weight, height, blood pressure and waist and hip perimeters. Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared. Fasting blood samples were obtained and used to measure ALT and AST, gamma-glutamyl transpeptidase (GGT), total cholesterol, HDL-cholesterol, TG, glucose, HbA1c and insulin. IR was calculated using the homeostatic model assessment (HOMA-IR) index[426]. Hepatic histopathological analysis was performed according to the scoring system of Kleiner et al[427]. Four histopathological features were semi-quantitatively evaluated: grade of steatosis (0, <5%; 1, 5%-30%; 2, >30%-60%; 3, >60%), lobularinflammation (0, no inflammatory foci; 1, <2 inflammatory foci per 200x field; 2, 2-4 inflammatory foci per 200x field; 3, >4 inflammatory foci per 200x field), hepatocellular ballooning (0, none; 1, few balloon

	All patients	Hepatocellular injury	Cholestatic injury	Mixed injury
n	21	9	7	5
Age (years)	49.3 ± 19.6	49.7 ± 19	47.7 ± 18.1	51.8 ± 26.8
Gender (F/M)	10/11	5/4	3/4	2/3
ALT (U/L)	438.8 ± 428.7	163.1 ± 109.5	722.4 ± 528.8	314.0 ± 89.8
AST (U/L)	324.9 ± 393.7	123.7 ± 58.8	568.2 ± 514.9	168.4 ± 70.2
GGT (U/L)	386.5 ± 433.1	744.5 ± 646.2	38.9 ± 35.6	296.2 ± 268.4
ALP (U/L)	360.8 ± 419.2	655.6 ± 617.1	143.2 ± 72.2	339.8 ± 194.3
TBIL	7.5 ± 6.0	6.6 ± 5.4	8.4 ± 7.4	7.3 ± 4.8
Severity, n (%)				
Mild	6 (28.6)	3 (33.3)	2 (28.6)	1 (20)
Moderate	11 (52.4)	3 (33.3)	5 (71.4)	3 (60)
Severe	3 (14.3)	2 (22.2)	0 (0)	1 (20)
Fatal	1 (4.8)	1 (11.1)	0 (0)	0 (0)

Table 4.1 Characteristics of DILI patients. Abbreaviations: ALT, Alaine aminotransferase; AST, Aspartate aminotransferase; GGT, Gamma glutamyl transferase; ALP, Alkaline phosphatase; TBIL, Total bilirubin. Data are expressed as mean ± SD.

cells; 2, many cells/prominent ballooning), and stage of fibrosis (from 0, none to 4, cirrhosis). Simple steatosis (SS) was defined as the presence of at least 5% of steatotic hepatocytes with or without mild lobular or portal inflammation but in the absence of features of hepatocellular injury (ballooning, apoptosis or necrosis) and fibrosis. On the other hand, minimal criteria for the histological diagnosis of definite NASH included the combined presence of grade 1 steatosis, hepatocellular injury and lobular inflammation with or without fibrosis.

4.1.4 Cholestasic liver diseases

PBC liver samples were obtained from the Biodonostia Health Research Institute-Donostia University Hospital and from the Hospital Clinic in Barcelona. Each patient signed an informed consent document. PBC liver samples were obtained by percutaneous biopsy (n=7). The diagnosis of PBC was established by liver biopsy with characteristic features of the disease and presence of AMAs. Liver samples from children with BAT (n=9), ALS (n=9), and liver disease associated with total parenteral nutrition (TPN) (n=9) were obtained at the time of transplantation. The diagnosis of biliary atresia was made on clinical, laboratory, radiological, and histopathological findings. Heterozygous mutations in *JAG1* gene were demonstrated in all children with ALS. The age of the patients ranged from 22 months to 5 years at the time of transplantation.

4.1.5 Cirrhosis

Alcoholic and viral cirrhotic samples were provided by Dr. Erica Villa and were obtained from 47 patients with liver cirrhosis detected during surveillance They had preserved liver function and corresponded to BCLC stage A (n=34) and B (n=13).

	NAFLD
n	21
Age (years)	50.2 ± 15.2
BMI (kg/m²)	29.6 ± 3.9
Gender (F/M)	17/4
ALT (IU/L)	24.2 ± 16.8
AST (IU/L)	20.4 ± 7.6
GGT (IU/L)	49.9 ± 50.2
HOMA-IR	2.7 ± 1.9
Total cholesterol (mg/dl)	212 ± 46.1
HDL-cholesterol (mg/dl)	48.4 ± 16.2
Triglyceride (mg/dl)	159.5 ± 72.3
Liver status (n)	
Grade of steatosis: 0/1/2/3(n)	0/15/5/1
NASH (%)	3 (14.3%)
Grade of fibrosis: 0/1/2/3/4(n)	19/2/0/0/0

Table 4.2 Characteristics of NAFLD patients from Santa Cristina Hospital. Abbreviations: BMI, body mass index; HDL, high density lipoprotein; HOMA-IR, homeostatic model assessment-insulin resistance. Data are expressed as mean ± SD.

4.1.6 HCC

Surgically resected liver specimens of 22 patients with HCC (10 Hepatitis C, 10 alcoholic steatohepatitis and 2 NASH) were examined. The Basque Research Biobank (http:// www.biobasque.org) provided the data and type of biospecimen. For the bad and better prognosis analyses we used samples from 47 patients with liver cirrhosis and HCC detected during surveillance. They had preserved liver function and corresponded to BCLC stage A (n=34) and B (n=13). These patients are part of another study, reported elsewhere (Gut, submitted). All tissues samples from this study were from US-guided liver biopsy of HCC lesions. In brief, compensated patients with liver cirrhosis under

	All patients	Simple steatosis	NASH
n	141	116	25
Age (years)	39 ± 10	40 ± 10	39.7 ± 8.3
BMI (kg/m²)	44.9 ± 5.4	44.7 ± 5.3	46.1 ± 5.9
Gender (F/M)	124/17	102/14	22/3
ALT (IU/L)	33.0 ± 20.3	31.1 ± 19.9	42.3 ± 20.4*
AST (IU/L)	24.2 ± 13.2	22.7 ± 11.1	31.4 ± 15.1*
GGT (IU/L)	42.2 ± 36.3	38.9 ± 35.4	57.8 ± 37.7*
HOMA-IR	4.6 ± 4.2	4.2 ± 4.1	6.0 ± 3.8*
Total cholesterol (mg/dl)	5.6 ± 1.1	216 ± 42.4	238 ± 51.5*
HDL-cholesterol (mg/dl)	1.4 ± 0.3	54.2 ± 14.4	53.5 ± 10.9
Triglyceride (mg/dl)	1.8 ± 1.1	146 ± 78.3	218 ± 177.5*
Liver status (n)			
Grade of steatosis: 0/1/2/3(n)	0/52/37/52	0/50/31/35	0/2/6/17
NASH (%)	25 (17.7%)	0	25
Grade of fibrosis: 0/1/2/3/4(n)	6/130/5/0/0	5/107/4/0/0	1/23/1/0/0

Table 4.3 Characteristics of the morbidly obese patients from Nice Hospital. Data are mean ± SD and compared using the non-parametric Mann Whitney test: *p < 0.05 compared with "Simple steatosis".

ultrasonographic surveillance for HCC, when first identified as having developed a HCC lesion underwent a a dedicated imaging protocol (2 computed tomography exams 6-weeks apart in a absence of any other therapy to evaluate growth speed) and an US-guided liver biopsy (to define transcriptomic characteristics of tumor). After the 2nd computed scan, patients underwent therapy according to internationally accepted guidelines. Patients were divided in 4 quartiles according to HCC volume doubling time and survival evaluated according to these quartiles. Patients of the first quartile (Doubling time< 53 days) had a definitively worse prognosis that those of the other quartiles (median survival 11 months vs 41, 42 and 47 months, respectively).

4.2 ANIMAL EXPERIMENTS

All animal experimentation was conducted in accordance with Spanish Guide for the Care and use of Laboratory animals, and with International Animal Care and Use of Committee. Mice were housed in a temperature-controlled animal facility accredited AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) with water and *libitum*.

Liver and serum samples were harvested, snap-frozen in liquid nitrogen and stored at -80ºC for subsequent analysis. The animals used for the experimentation were:

- MCJ KO and WT (C57BL/6J) mice: 3 month-old male were used for NAFLD and DILI studies and subjected to APAP overdose and MCDD experiments.

- *Phb1* KO and WT (C57BL/6J) mice: 3 month-old male were used for cholestasis studies and subjected to BDL experiments, 5 to 6-month-old male were used for HCC *in vivo* studies and 8-month-old male were used for HCC *in vitro* studies.

- Athymic C57BL/6J nude mice: 3 month-old male were used for HCC-xenograft studies.

4.2.1 APAP overdose

APAP was dissolved in phosphate buffered saline (PBS) at 37ºC and injected intraperitoneally at a dose of 360 mg/kg to WT and MCJ KO mice (n=6). Animals were sacrificed at 6, 24 and 48 hours and livers were rapidly split into several pieces, some were snap frozen for subsequent RNA or protein extraction, others were formalin fixed for histology and IHC. Blood for serum analysis was also collected.

MCJ silencing: WT mice were subjected to APAP 360 mg/kg intraperitoneal injection and 6 or 24 hours later were separated into two groups (n=5) and subjected to a single 150 µl tail vein injection of a 0.4 µg/µl solution of control or specific small interfering RNA (siRNA) against MCJ (siMCJ) (sequences are shown in Table 4.5) using Invivofectamine 3.0 reagent (Thermo Fisher Scientific). At 48 hours mice were sacrificed.

NAC treatment: WT mice were subjected to APAP 360 mg/kg intraperitoneal injection and 6 hours later were separated into two groups (n=4) and one of them was subjected to a single 200 µl intraperioteneal injection of a solution of NAC 150µg/µl. At 48 hours mice were sacrificed.

4.2.2 MCDD

WT animals were fed with a methionine (0.1%) and choline (0%) deficient diet for 4 weeks. 1 week after the beginning of the diet mice were separated into two groups (n=11) and subjected to tail vein 150 µl injections of a 0.4 µg/µl solution of control or specific siRNA against MCJ (siMCJ) (sequences are shown in Table 4.5) once a week during the remaining 3 weeks using Invivofectamine 3.0 reagent (Thermo Fisher Scientific). At the time of sacrifice, livers were rapidly split into several pieces, some were snap frozen for subsequent RNA or protein extraction, others were formalin fixed for histology and immunohistochemistry or freshly used to measure FA β-oxidation, DNL and mitochondrial respiration. Blood for serum analysis was also collected.

4.2.3 BDL

WT and *Phb1* KO mice (n=5) were subjected to complete BDL as described. In brief, mice were anesthetized with isoflurane (1.5% isoflurane in O_2), the abdomen was opened and the with the help of forceps the common bile duct was separated carefully from the flaking portal vein and hepatic artery and subsequently, a suture was placed around the bile duct and secured with a surgical knot. The abdomen was closed and animals were sacrificed at 3 and 7 days.

PHB1 and OPA1 silencing: WT mice underwent BDL and 3 days after received via tail vein injection, either 200 µl of a 0.75 µg/µl solution of control (pSM2c Dharmacon) or PHB1 and OPA1 specific shRNA (Dharmacon) using jetPEI reagent (Polyplus) (n=5). Animals were sacrificed 14 days after BDL and livers were rapidly split into several pieces, some were snap frozen for subsequent RNA or protein extraction, others were formalin fixed for histology and IHC. Blood for serum analysis was also collected.

Parthenolide: *Phb1* KO mice were subjected to vehicle or parthenolide 3 mg/kg intraperitoneal injections 24h and 1h before BDL (n=5). Animals were sacrificed 3 days after the surgery.

HDAC4 silencing: *Phb1* KO animals received 1 day before and 2 days after BDL via tail vein injection, 100 µl of a 25 µM solution of control or specific siRNA against HDAC4 (siHDAC4) (sequences are shown in Table 4.5) (n=3) using jetPEI reagent (Polyplus). Animals were sacrificed at 7 days.

4.2.4 Isolation of biliary trees

Biliary trees were isolated from WT and *Phb1* KO mice (n=2) after BDL. In brief, BDL was performed and 7 days after livers were digested with collagenase as described in section 4.3.1.1. Once digested, livers were excised, transferred into a Petri dish containing PBS and disrupted with the help of forcepts to allow the separation of biliary trees. Bilary trees were collected for subsequent RNA extraction.

4.2.5 Parthenolide *in vivo* treatment

5 to 6-months old *Phb1* KO mice presenting liver fibrosis were selected and randomly assigned to two different experimental groups (n=3). Parthenolide was intraperitoneally injected at a dose of 3 mg/kg twice a week for 2 weeks. At the time of sacrifice, livers were rapidly split into several pieces, some were snap frozen for subsequent RNA or protein extraction and others were formalin fixed for histology and IHC.

4.2.6 MLN4924 *in vivo* treatment

5 to 6-months old *Phb1* KO mice bearing liver tumors were selected and randomly assigned to two different experimental groups (n=6). One group was subjected to subcutaneous injection of MLN4924 (60 mg/kg body weight) and the other to vehicle injections, once a week for 8 weeks, following the Millennium Corporation communication. Tumor size was evaluated by *in vivo* high-frequency ultrasound fortnightly. The Vevo 770 high-frequency Ultrasound system (VisualSonics Inc.) was used employing a 40-MHz probe (Scanhead RMV-704, VisualSonics Inc.). During imaging sessions, mice were kept under anesthesia with 1.5% isoflurane in oxygen and restrained on a heated stage (THM-150, Indus Instruments). Images of the liver were acquired through the ventral body wall in transverse and sagittal orientations in each animal fortnightly during 8 weeks. The liver parenchyma was examined for echogenicity, homogeneity, presence or absence of nodules, echostructure and border definition. The size of the nodules was determined by caliper measurement of the longest diameter in the transverse view of the liver.

4.2.7 HepG2 HCC xenograft

5×10^6 HepG2 cells were subcutaneously injected into the right flank of 10 male athymic C57BL/6J nude mice. Cells were cultured as described in section 4.3.1.3, trypsinized and resuspended in 100ml of a DMEM/matrigel solution (70ml DMEM and 30ml matrigel) for injection. One week after cell inoculation, when the tumors were palpable, animals were divided into two groups (n=5) and subjected to control or specific siRNA against Nedd8 (siNedd8) (sequences are shown in Table 4.5) 50μM injections using jetPEI™ reagent (Polyplus), three times per week. Tumor volume was measured thrice a week during the treatment and prior to necropsy. Tumor volumes were estimated with a caliper and calculated according to the following formula: Tumor volume (cm^3)=(length [mm]×width^2 [cm^2])/2.

4.3 CELL EXPERIMENTS

4.3.1 Cell isolation and maintenance

4.3.1.1 Primary mouse hepatocytes

All animals used for primary hepatocytes isolation were 3-months-old except for neddylation experiments' *Phb1* KO mice, that were 8-months-old. Primary hepatocytes from WT, MCJ KO and *Phb1* KO mice were isolated by perfusion with collagenase type IV (Worthington). In brief, mice were anesthetized with isoflurane (1.5% isoflurane in O_2), the abdomen was opened and a catheter was inserted into the vena. Liver was perfused with buffer A [1X PBS, 5 mM EGTA] (37°C, oxygenated) and portal vein was cut. Subsequently, liver was perfused with buffer B [1X PBS, 1mM $CaCl_2$, collagenase type I (Worthington)] (37°C, oxygenated). After the perfusion, liver was placed in a petri dish containing buffer C [1X PBS, 2mM $CaCl_2$, 0.6% bovine serum albumin (BSA)] and disaggregated with forceps. Digested liver was filtered through sterile gauze; hepatocytes were collected and washed twice in buffer C (300 rpm, 3 minutes, 4ºC). Supernatant was removed and hepatocytes were resuspended in fresh 10%-fetal bovine serum (FBS; Gibco) Minimun Essential Medium (MEM; Gibco) containing penicillin (100 U/ml), streptomycin (100 U/ml), and glutamine (2 mM) (PSG; Invitrogen). Cell viability was validated by trypan blue exclusion test and more than 80% of viability was considered for the experiments.

Isolated hepatocytes were seeded over collagen-coated culture dishes at a density of 7600 cells/mm^2 in 10% FBS MEM supplemented with 1% PSG and maintained in a 5% CO_2-95% air incubator at 37°C. After 2 hours of attachment, medium was removed and replaced with fresh 0% or 5%-FBS medium, and different treatments were perfomed after 2 additional hours of incubation or overnight incubation.

4.3.1.2 BCLC3 and BCLC5 cell lines

Barcelona Clinic Liver Cancer (BCLC) human cell lines BCLC3 and BCLC5 were isolated from resected HCCs from patients with hepatitis C viral infections. These cell lines were kindly provided to us by Dr. Jordi Bruix and Dr Loreto Boix (BCLC group, Hospital Clinic, Barcelona, Spain). Cells were maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) media supplemented with 10% FBS, 1% PSG, 1% Sodium pyruvate (HyClone) and 1% Non-essential amino acids (Gibco) at 37ºC in a humidified atmosphere of 5% CO_2-95% air.

4.3.1.3 HepG2 cell line

The human hepatoma cell line HepG2 was purchased from the American Type culture Collection (ATCC). Cells were cultured in 10% FBS Dulbecco's Modified Eagle Medium (DMEM) supplemented with

1% PSG and maintained in a 5% CO_2-95% air incubator at 37°C.

4.3.2 Cell treatments

Primary hepatocytes and tumor cell lines were subjected to different treatments in the present study. Reagents, concentrations and the appropriated conditions employed for every compound are listed in Table 4.4.

- Primary hepatocytes: 500,000 cells were seeded in 6-multiwell plates. For DCA and APAP experiments, cells were cultured overnight in 0% FBS MEM 1% PSG medium and treated the day after. For DCA experiments, HDAC inhibitors were added the night before. For APAP experiments, rotenone and malonate were added 3 hours before APAP. For OA experiments cells were incubated overnight with 0% FBS MEM 1% PSG medium containing OA and rotenone was added 6 hours before cell harvesting. For MLN4924 experiments, hepatocytes were changed to 5% FBS MEM 1% PSG once attached and treated after 2 hours of additional incubation.

- Tumor cells: BCLC3, BCLC5 and HepG2 cells were seeded in 6-multiwell plates (100,000 per well for BCLC3 and BCLC5 cell lines and 175,000 for HepG2 cell line) in 10% FBS medium. Once cells were attached medium was changed to 5% FBS and MLN4924 treatment was initiated the next morning.

4.3.3 Gene silencing

siRNA constructs were designed by Qiagen or Sigma and annealed according to manufactuter´s instructions. Negative controls were included in each assay by using non-related siRNA (siRNA control). Nucleotide sequences designed for each specific siRNA are detailed in Table 4.5. Gene silencing efficiency was confirmed by Western blott and/or RNA expression analysis. We used two different protocols for the transfection:

Primary hepatocytes were transfected with siRNA for gene silencing using Jetprime reagent (Polyplus) according to manufacturer´s instructions. Protocol is described as follows: 5 µl of Jetprime and 10 µl of siRNA 20 µM were diluted separately in 200 µl of Jetprime buffer, incubated for 5 minutes at RT and mixed. Mix was incubated for 20 minutes at RT to allow the formation of siRNA-Jetprime complexes. For each transfection, 200 µl of the mix were added to 60 mm culture plates containing 500,000 cells in 2 ml of 10% FBS MEM medium supplemented with 1% PSG. The final concentration of the siRNA was 100 nM. The mix was left overnight and then the medium was replaced for fresh 5% FBS MEM 1% PSG. For DCA experiments, the mix was left 7 hours and the medium was replaced for fresh 0% FBS MEM 1% PSG. Afterwards, different cell treatments were performed as described in Table 4.4.

Tumor cell lines were transfected with siRNA for gene silencing using Lipofectamine 2000 (Invitrogen) according to manufacturer´s instructions. Protocol is described as follows: 5 µl of Lipofectamine 2000 were diluted in 500 µl of OptiMEM (Gibco) for 5 minutes at RT and mixed with the same volume of OptiMEM containing 15 µl of siRNA (20 nM). Mix was incubated for 20 minutes at RT to allow the formation of the siRNA-Lipofectamine complexes. Cells (200,000 for BCBL3 and BCLC5 cell lines and 350,000 for HepG2 cell line) were resuspended in 2 ml of 10% FBS 1% PSG medium and placed in 60 mm culture plates containing the siRNA-Lipofectamine complexes previously formed. The final concentration of siRNA was 100 nM. Mix was left overnight and then medium was replaced for fresh 10% FBS 1% PSG medium. 8 hours later, silencing assay was repeated

Table 4.4 Appropriated conditions of the different cell treatments performed. Abbreviations: DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; HDAC, histone deacetylase; NAE1, Nedd8 activating enzyme; PBS, phosphate buffered saline.

Compound	Dose	Vehicle	Function	Supplier	% FBS Medium
Acetaminophen (APAP)	10mM	PBS	Paracetamol	Sigma	0
Apicidin	1nM	DMSO	HDAC3 inhibitor	Sigma	0
Cycloheximide (CHX)	50µg/ml	Ethanol	Protein synthesis inhibitor	Sigma	10
Deoxycholic acid (DCA)	100µM	Ethanol	Bile acid	Sigma	0
Malonate	20mM	Water	Complex II inhibitor	Sigma	0
MLN4924	3µM	DMSO	NAE1 inhibitor	Millenium	5
Mocetinostat	0.15nM	DMSO	HDAC1, 2 and 3 inhibitor	Selleckchem	0
Oleic acid (OA)	400µM	Ethanol	Fatty acid	Sigma	0
Parthenolide	2.5µM	DMSO	HDCA1 inhibitor	Sigma	0
PCI34051	10nM	DMSO	HDAC8 inhibitor	Selleckchem	0
Rocilinostat	50nM	DMSO	HDAC6 inhibitor	Selleckchem	0
Rotenone	0.1µM	DMSO	Complex I inhibitor	Sigma	0
Tichostatin A (TSA)	3µM	Ethanol	HDAC pan inhibitor	Sigma	0

over the attached cells. In total, tumor cells were silenced twice during a 48-hours period (once every 24 hours).

4.3.4 Cell transfection

Primary hepatocytes were transfected with cDNA plasmids for gene expression using Jetprime reagent (Polyplus) according to manufacturer´s instructions. Every assay was perfomed in triplicate. Plasmids used for transfection were: pcDNA3-LacZ (Invitrogen) and pLNCX1 (provided by Dr. Carracedo) as negative controls, shMCJ and HA-MCJ (provided by Dr. Anguita), pCMV6-HDAC4 (Origene), pcDNA3-FLAG-LKB1 (Addgene), pLNCX1-HA-Akt (provided by Dr. Carracedo), NEDP1-V5 (provided by Dr. Xirodimas) and His_6-Nedd8 (provided by Dr. Rodríguez). Transient transfection protocol is described as follows:

DNA-Jetprime complex formation: 4 µl of Jetprime and 2 µg of DNA were diluted separately in 200 µl of Jetprime buffer, incubated for 5 minutes at RT and mixed. Mix was incubated for 20 minutes at RT to allow the formation of siRNA-Jetprime complexes. For each transfection, 200 µl of the mix were added to 60 mm culture plates containing 500,000 cells in suspension in 2 ml of 10% FBS MEM medium supplemented with 1% PSG. For MCJ and HDAC4, mix was left 7 hours, then medium was replaced for fresh 0% FBS 1% PSG medium and different cell treatments were performed next day as described in table 4.4. For NEDP1, mix was left overnight and medium was replaced for fresh 10% FBS 1% PSG medium. FLAG-LKB1 and HA-Akt were co-transfected with His_6-Nedd8 overnight and then medium was replaced for fresh 10% FBS medium supplemented with antibiotics. 24 hours after the transfection, cells were harvested. Transfection efficiency was confirmed by Western blotting and/or RNA expression analysis.

4.3.5 Viral infection

For HuR knockdown and overexpression HepG2 and BCLC3 cell lines were treated with short-hairpin lentiviral particles against HuR (shHuR) and HuR adenoviral constructs (AdHuR) respectively.

For lentiviral infections, around 500,000 BCLC3 and 850,000 HepG2 cells were seeded in 100 mm culture plates and treated with lentiviral particles against HuR kindly provided by Dr. Wodhoo (amount added determined by titration) in the presence of Polybrene (1.1000) (Sigma). 12 hours later, medium was changed to fresh medium. After 72 hours, 50,000 BCLC3 and 70,000 HepG2 infected cells were seeded in M6 multiwell culture plates. The day after medium was changed to 5% FBS and 2-3 hours later cells were treated as described in table 4.4.

For adenoviral infections around 500,000 BCLC3 and 850,000 HepG2 cells were seeded in 100

Table 4.5 siRNA sequences used for target silencing.

Gene name	Species		Sequence
siControl	*Homo sapiens*	Sense	5'-UUCUCCGAACGUGUCACAU-3'
		Antisense	5'-AUGUGACACGUUCGGAGAA-3'
siControl	*Mus musculus*	Sense	5'-AAUUCUCCGAACGUGUCACGU-3'
		Antisense	5'-ACGUGACACGUUCGGAGAAUU-3'
siAkt	*Mus musculus*	Sense	5'-GCUACUUCCUCCUCAAGAA-3'
		Antisense	5'-UUCUUGAGGAGGAAGUAGC-3'
siHDAC4	*Mus musculus*	Sense	5'-CAGGCGTGGGTTTCAATGTCA-3'
		Antisense	5'-GGCGUGGGUUUCAAUGUCATT-3'
siLKB1	*Mus musculus*	Sense	5'-GGGCGGUCAAGAUCCUCAA-3'
		Antisense	5'-UUGAGGAUCUUGACCGCCC-3'
siMCJ	*Mus musculus*	Sense	5'-AAGCGAGAGGCUAGUCUUATT-3'
		Antisense	5'-UAAGACUAGCCUCUCGCUUAC-3'
siNedd8	*Homo sapiens*	Sense	5'-GCCCAGUAAUGUAUGUCUA-3'
		Antisense	5'-UAGACAUACAUUACUGGGCAU-3'
siNedd8	*Mus musculus*	Sense	5'- CAUCUACAGUGGCAAGCAA -3'
		Antisense	5'- UUGCUUGCCACUGUAGAUGAG-3'
siOPA1	*Mus musculus*	Sense	5'- CAGUAGACAUCAAGCUUAAAC-3'
		Antisense	5'-UUAAGCUUGAUGUCUACUGUG-3'
siPHB1	*Mus musculus*	Sense	5'-AGAGCGAGCGGCAACAUUU-3'
		Antisense	5'-AAAUGUUGCCGCUCGCUCU-3'

mm tissue culture plates and treated with HuR adenoviral particles or matched Green fluorescent protein (GFP) control kindly provided by Dr. Wodhoo (amount added determined by titration). 12 hours later, medium was changed to fresh medium and 50,000 BCLC3 and 70,000 HepG2 infected cells were seeded in M6 multiwell culture plates. The day after medium was changed to 5% FBS and 2-3 hours later cells were treated as described in table 4.4. Since AdHuR and its matched control contain GFP, the infection effectiveness was determined by fluorescence microscopy.

4.4 RNA EXTRACTION AND PROCESSING

4.4.1 RNA isolation

Total RNA was isolated from mouse livers and biliary trees, cultured primary mouse hepatocytes and human cell lines using Trizol reagent (Invitrogen) according to manufacturer's instructions. RNA concentration and purity was determined in the Nanodrop ND-1000 spectrophotometer (Thermo Scientific).

4.4.2 Quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR)

1 to 2 µg of the obtained RNA were treated with DNAse I (Invitrogen) and cDNA was synthesized with M-MLV retrotranscriptase (Invitrogen) in the presence of random primers and RNAseOUT (Invitrogen). For the polymerase chain reactions (PCRs) performed using the BioRad iCycler iQ5 and iQ SYBR Green Super Mix (BioRad), 5µl of cDNA and specific primers were used, in a total reaction volume of 20 µl. For the PCRs performed using the Viia™ 7 System (Applied Biosystems) and SYBR® Select Master Mix (Applied Biosystems), 1.5 µl of cDNA and specific primers were used, in a total reaction volume of 6.5µl. All reactions were performed in triplicate. PCR conditions or these primers were optimized, and 40 cycles with a melting temperature of 60ºC, were used. Primers were designed using Primer3 software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and synthesized by Sigma-Aldrich. The primers used for each gene are provided in Table 4.6. After checking the specificity of the PCR products with the melting curve, Ct values were extrapolated to a standard curve performed simultaneously with the samples and data was then normalized to the expression of a housekeeping gene (GAPDH and 9S).

Table 4.6 Sequence of primers used for RT-qPCR.

Gene name	Symbol	Species		Sequence
9S ribosomal RNA	9S	*Mus musculus*	Forward	5'-GACTCCGGAACAAACGTGAGG-3'
			Reverse	5'-CTTCATCTTGCCCTCGTCCA-3'
ATP Binding Cassette Subfamily D Member 1	ABCD1	*Mus musculus*	Forward	5'-AGTGCCATCCGCTACCTAGA-3'
			Reverse	5'-CAGGGTTTCGAAGTCGTCCA-3'
Acyl-CoA Dehydrogenase, Long Chain	ACADL	*Mus musculus*	Forward	5'-GTCCGATTGCCAGCTAATGC-3'
			Reverse	5'-CACAGGCAGAAATCGCCAAC-3'
Acyl-CoA Dehydrogenase, C-4 To C-12 Straight Chain	ACADM	*Mus musculus*	Forward	5'-TCAAGATCGCAATGGGTGCT-3'
			Reverse	5'-GCTCCACTAGCAGCTTTCCA-3'
Protein Kinase B Alpha	Akt1	*Mus musculus*	Forward	5'- AATGTGGGCTCATGGGTCTG -3'
			Reverse	5'- AGAGGGAGAGGGCCAGTTAG -3'
Protein Kinase B Alpha	Akt1	*Homo sapiens*	Forward	5'-ATCCTGGTCCGTCTTCCTC-3'
			Reverse	5'-CTTCCCTAAGCCCCTGGTGA-3'
Albumin	ALB	*Mus musculus*	Forward	5'-GACTTTGCACAGTTCCTGGATACA-3'
			Reverse	5'-TTGTGGTTGTGATGTGTTTAGGCTA-3'
Adenine Nucleotide Translocator 1	ANT	*Mus musculus*	Forward	5'-GCCAGCAAACAGATCAGTGC-3'
			Reverse	5'-AGTGGGGAAGTACCGGATCA-3'
BCL2 Associated X, Apoptosis Regulator	Bax	*Mus musculus*	Forward	5'- TGCAGAGGATGATTGCTGAC-3'
			Reverse	5'-GATCAGCTCGGGCACTTTAG-3'
B-cell lymphoma 2	BCL2	*Mus musculus*	Forward	5'-CTGCACCTGACGCCCTTCACC-3'
			Reverse	5'-CACATGACCCCACCGAACTCAAAGA-3'
Chemokine (C-C motif) ligand 2	CCL2	*Mus musculus*	Forward	5'-AGATGCAGTTAACGCCCCAC-3'
			Reverse	5'-ACCCATTCCTTCTTGGGGTC-3'
Cytokeratin-19	CK19	*Mus musculus*	Forward	5'-AGGCGAGCATTGTCAATCTG-3'
			Reverse	5'-GTGAAGATCCGCGACTGGT-3'

Collagen Type I Alpha 1	COL1A1	*Mus musculus*	Forward	5’-AGTCGCTTCACCTACAGCAC-3'
			Reverse	5’-GAGGGAACCAGATTGGGGTG-3'
Carnitine palmitoyltransferase 1A	CPT1	*Mus musculus*	Forward	5’-GACTCCGCTCGCTCATTCC-3'
			Reverse	5’-GAGATCGATGCCATCAGGGG-3'
C-X-C Motif Chemokine Ligand 1	CXCL1	*Mus musculus*	Forward	5’-CTGGGATTCACCTCAAGAACAT-3'
			Reverse	5’-CAGGGTCAAGGCAAGCCTC-3'
Cyclin D1	CCND1	*Mus musculus*	Forward	5'-GCCTCTAAGATGAAGGAGACC-3'
			Reverse	5'-ATTTTGGAGAGGAAGTGTTCG-3'
Cytochrome P450 Family 7 Subfamily A Member 1	CYP7A1	*Mus musculus*	Forward	5'-GAGCGCTGTCTGGGTCACGG-3'
			Reverse	5'-GCCAGCCTTTCCCGGGCTTT-3'
Epithelial cell adhesion molecule	Epcam	*Mus musculus*	Forward	5’-CCTGCGAAGGGTTACTGCTT-3'
			Reverse	5’-GAGTACCACACTGGACCTGC-3'
Fatty Acid Transport Protein 2	FATP2	*Mus musculus*	Forward	5’-CCGCAGAAACCAAATGACCG-3'
			Reverse	5’-TGCCTTCAGTGGATGCGTAG-3'
Fructose-Bisphosphatase 1	FBP1	*Mus musculus*	Forward	5’-GTCTGTTTCGATCCCCTTGA-3'
			Reverse	5’-TCCAGCATGAAGCAGTTGAC-3'
Farnesoid X-Activated Receptor	NR1H4	*Mus musculus*	Forward	5'-GGCCTCTGGGTACCACTACA-3'
			Reverse	5'-AAGAAACATGGCCTCCACTG-3'
Glyceraldehyde-3- phosphate dehydrogenase	GAPDH	*Mus musculus*	Forward	5'-CGTCCCGTAGACAAAATGG-3'
			Reverse	5'-TTGATGGCAACAATCTCCAC-3'
Histone deacetylase 4	HDAC4	*Mus musculus*	Forward	5’-GGAAACGAGCTTGAGCCTCT-3'
			Reverse	5’-CTCAGCAGGTTTGACGCCTA-3'
Hepatocyte growth factor	HGF	*Mus musculus*	Forward	5’-TGCTCCTCCCTTCCCTACTC-3'
			Reverse	5’-ATGCCGGGCTGAAAGAATCA-3'
Hexokinase 2	HK2	*Mus musculus*	Forward	5’-GGGTAGCCACGGAGTACAAA-3'
			Reverse	5’-TGGATTGAAAGCCAACTTCC-3'
Hexokinase 4	HK4	*Mus musculus*	Forward	5’-CTTTCCAGGCCACAAACATT-3'
			Reverse	5’-TGAGTGTTGAAGCTGCCATC-3'
Interleukin 6	IL-6	*Mus musculus*	Forward	5’-AAGTGAGCAGATAGCACAGTTGG-3'
			Reverse	5’-GCTATGAAGTTCCTCTCTGCAAGTA-3'
Methylation J-controlled protein	MCJ	*Mus musculus*	Forward	5’ -ACGCCGACATCGACCACACAG-3’
			Reverse	5’-AATCTTCCTTGCTGTTGCCGTC-3’
Methylation J-controlled protein	MCJ	*Homo sapiens*	Forward	5’-ACGCCGACATCGACCACACAG-3'
			Reverse	5’-AATCTTCCTTGCTGTTGCCGTG-3'
Nedd8 Activating Enzyme 1	NAE1	*Homo sapiens*	Forward	5’-TTGTGGCCAAAGAGGGTCAA-3’
			Reverse	5’-ATGATTACCCACAGCGGCAG-3
Neural precursor cell expressed, developmentally down-regulated 8	Nedd8	*Mus musculus*	Forward	5’-CAGCAGCGGCTCATCTACAG-3’
			Reverse	5’-CAGGGCAAGGAGGTAAACGG-3'
Neural precursor cell expressed, developmentally down-regulated 8	Nedd8	*Homo sapiens*	Forward	5’-CTACAGACAAGGTGGAGCGAA-3’
			Reverse	5’-CTCCTCTCAGAGCCAACACC -3'
Nuclear Factor, Erythroid 2 Like 2	NRF2	*Mus musculus*	Forward	5’-TGTAGGGTGGGGGTACAAAG-3'
			Reverse	5’-GAATCGGCGCTAAGGAACCC-3'
Phosphofructokinase, Liver Type	PFKL	*Mus musculus*	Forward	5’-CATATATGTGGGGGCCAAAG-3'
			Reverse	5’-GACACACAGGTTGGTGATGC-3'
PPARG Coactivator 1 Alpha	PPARGC1A	*Mus musculus*	Forward	5’AGACAGGTGCCTTCAGTTCAC-3’
			Reverse	5’-ACCAGAGCAGCACACTCTATG-3’

Prohibitin-1	PHB	*Mus musculus*	Forward	5'-GCATTGGCGAGGACTATGA-3'
			Reverse	5'-AGCTCTCGCTGGGTAATCAA -3'
Prohibitin-1	PHB	*Homo sapiens*	Forward	5'-ATGATGTGCACTTTGGGCGA-3'
			Reverse	5'-ACCACAATGTCCTGCACTCC-3'
Prothymosin, Alpha	PTMA	*Mus musculus*	Forward	5'-CAGCTTTATCGCCAGCGTCC-3'
			Reverse	5'-AGTCCTTGGTGGTGATTTCG-3
Serine-threonine kinase 11, Liver Kinase B1	STK11	*Mus musculus*	Forward	5'- GTCAGCTGGGGTCACACTTT -3'
			Reverse	5'- TGGTGAAGTCTCCTCTCCCA -3'
Serine-threonine kinase 11, Liver Kinase B1	STK11	*Homo sapiens*	Forward	5'-CGAGGGCAGCTGATGTCGGT-3'
			Reverse	5'-CCGCCCTGCGGCATAAGGTCT-3'
Transforming Growth Factor Beta 1	TGFβ1	*Mus musculus*	Forward	5'-TGGTTGTAGAGGGCAAGGAC-3'
			Reverse	5'-TTGCTTTCAGCTCCACAGAGA-3'
Tumor necrosis factor	TNF	*Mus musculus*	Forward	5'- CGTCAGCCGATTTGCTATCT-3'
			Reverse	5'-CGGACTCCGCAAAGTCTAAG -3'
Tumor necrosis factor receptor 2	TNFR2	*Mus musculus*	Forward	5'-GCCCAGGTTGTCTTGACACC-3'
			Reverse	5'-CACAGCACATCTGAGCCTTCC-3'
TNF-Related Apoptosis-Inducing Ligand	TNFSF10	*Mus musculus*	Forward	5'-CCAACGAGATGAAGCAGC-3'
			Reverse	5'-CCATCAGTGGAGTCCCAG-3'
Vascular endothelial growth factor A	VEGF	*Mus musculus*	Forward	5'-TCTCCTTACCCCACCTCCTG-3'
			Reverse	5'-ACACACAGCCAAGTCTCCTG-3'
X-Linked Inhibitor Of Apoptosis	XIAP	*Mus musculus*	Forward	5'-GGGGTTCAGTTTCAAGGACA-3'
			Reverse	5'-CGCCTTAGCTGCTCTTCAGT-3'
Alpha smooth muscle actin	αSMA	*Mus musculus*	Forward	5'-CATCTCCAAGAGTCCAGCACA--3'
			Reverse	5'-CTGACAGAGGCACCACTGAA-3'

4.5 PROTEIN EXTRACTION AND ANALYSIS

4.5.1 Total protein extraction

Cells were washed twice with PBS buffer and homogenized in lysis buffer (NaH_2PO_4 1.6 mM, Na_2HPO_4 8.4 mM, Triton X-100 0.1%, NaCl 0.1 M, 0.1% SDS, 0.5% sodium azide) supplemented with protease and phosphatase inhibitor cocktail (Roche). In the case if frozen liver tissue, approximately 50 µg of tissue was homogenized by using Precellys homogenizer in 1 ml lysis buffer for whole cell lysate. In both cases the lysates were centrifuged (13000 rpm, 30 min, 4ºC) and the supernatant (protein extract) was quantified for total protein content by the BioRad protein assay, or byBCA protein assay (Pierce) when measuring proteins with high fat content.

4.5.2 Subcellular protein extraction

- Cytosolic, membrane and nuclear lysates from both cells and frozen liver tissue samples were prepared as described by the manufacturer by using the Subcellular Proteome Extraction Kit (Calbiochem). The lysates were quantified for protein content by the BCA protein assay (Pierce).

- Mitochondrial lysates were prepared as described by the manufacturer by using the Mitochondria/Cytosol Fractionation Kit (Abcam). Basically, cells were trypsinized and frozen livers sprayed using a mortar cooled with liquid nitrogen, resuspended in cytosolic buffer, disrupted using dounce homogenizers (50 strokes on ice) and subjected to several centrifugations to obtain crude mitochondria pellets. Mitochondria were lysed using mitochondrial buffer and the lysates were quantified for protein content by the BCA protein assay (Pierce).

4.5.3 Western blotting

Protein extracts were boiled at 95ºC for 5 minutes in sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) sample buffer (250 mM Tris-HCl pH 6.8, 500 mM β-mercaptoethanol, 50% glycerol, 10% SDS, bromophenol blue). Appropriate amount of protein (5 to 30 µg), according with the specific protein abundance and antibody sensitivity, was separated by SDS-PAGE in 8%, 11% or 15% acrylamide gels (depending on the molecular weight of the protein), using Mini-PROTEAN Electrophoresis System (BioRad). Gels were transferred onto nitrocellulose membranes by electroblotting using Mini Trans-Blot cell (BioRad). Membranes were blocked with 5% nonfat dry milk in TBS pH 8.0 containing 0.1% Tween-20 (TBST-

0.1%), for 1 hour at room temperature, washed three times with TBST-0.1% and incubated overnight at 4ºC with commercial primary antibodies. Optimal incubation conditions are detailed in Table 4.7. Membranes were then washed three times with TBST-0.1% and incubated for 1h at room temperature in blocking solution containing secondary anti-rabbit or anti-mouse antibodies conjugated to horseradish peroxidase as detailed in Table 4.7. Immunoreactive proteins were detected by Western Lighting Enhanced Chemiluminiscence reagent (ECL, Perkin Elmer) or Clarity Western ECL Substrate (BioRad), and exposed to X-ray films (Amersham) in a Curix 60 Developer (AGFA). Bands were quantified by densitometry using the free image processing software ImageJ (http://rsbweb.nih.gov/ij).

4.5.4 BN-PAGE

Mitochondria were isolated as described in section 4.5.2 and stored at -80ºC. Purified mitochondria were solubilized in NativePAGE™ sample buffer (Invitrogen) containing 2% digitonin (Sigma). The suspension was kept on ice for 15 minutes and was then clarified by centrifugation at 20,000 × *g* for 30 minutes. Following centrifugation, protein concentration was determined by the BioRad protein assay and 9 µl of the resulting supernatant containing 30 µg of both membrane and water-soluble proteins was mixed with 1 µl of NativePAGE™ 5% G-250 sample additive. Samples were kept on ice all the time and loaded into Native PAGE 3-12% or 4-16% Bis-Tris 1.0mmx10well gels for native separation of protein complexes. Gels were run in the cold room at a constant voltage of 150V following manufacturer's instructions and transferred onto polyvinylidene difluoride (PVDF) membranes using the iBlot® blotting system (iBlot® transfer stacks and program P3: 20V, 7 minutes). Membranes were processed as described in section 4.5.

4.6 RESPIRATION ANALYSIS

Respiration analyses were performed in cells and isolated mitochondria using a Seahorse® Bioscience XF24-3 Extracellular Flux Analyzer. Oxidative phosphorylation was measured as the oxygen consumption rate (OCR), which is the rate change of dissolved O_2. Glycolysis was measured as the extracellular acidification rate (ECAR), which is the rate change of pH in medium immediately surrounding adherent cells. All measurements were performed at 37ºC. Basal respiration corresponds to OCR baseline measurements. ATP production rate was calculated by subtracting OCR values after ATP synthase inhibition to basal OCR values. Proton leak corresponds to the remaining OCR of the basal after subtracting ATP-coupled OCR.

4.6.1 Primary hepatocytes and human hepatoma cells

Primary WT, MCJ KO and *Phb1* KO mouse hepatocytes cells were seeded in collagen I coated XF24 cell culture microplates (Seahorse Bioscience), at 2.0 × 10^4 cells per well. BCLC5 cells were seeded in polyornithine (Sigma)-coated XF24 cell culture microplates (Seahorse Bioscience), at 2.0 × 10^4 cells per well. After 3 hours, 100 µl of growth media were added. The day after, growth medium was removed and replaced with 500 µl of assay medium prewarmed to 37°C, composed of DMEM without bicarbonate containing 1 mM sodium pyruvate, 2 mM L-glutamine, and cultured at 37°C in room air. Measurements of OCR and ECAR were performed after equilibration in assay medium for 1h. After OCR and ECAR baseline measurement, sequential injections were performed through ports in the XF Assay cartridges. The following pharmacologic inhibitors were used: oligomycin (1mM), an inhibitor of ATP synthase, which allows the measurement of ATP-coupled oxygen consumption through OXPHOS; carbonyl cyanide 4-trifluoromethoxy-phenylhydrazone (FCCP) (300 nM), an uncoupling agent that allows maximum electron transport, and therefore a measurement of the maximal OXPHOS respiration capacity; and rotenone (1 µM), a mitochondrial complex I inhibitor. Upon the sequential delivery of the inhibitors, changes in OCR were recorded.

For APAP experiments, APAP 10 mM was injected first. For neddylation experiments, at 3 hours after cell seeding, 100 µl of growth media with DMSO or MLN4924 and control or Nedd8 siRNA were added. 48 hours after, growth medium was removed and replaced with 500 µl of assay medium prewarmed to 37°C. The normalize data were expressed as pmol of O_2 per min or milli-pH units (mpH) per min, per µg protein for primary hepatocytes and viability measured by MTT assay for BCLC5 cells.

4.6.2 Isolated mitochondria

For mitochondrial respiration experiments, liver mitochondria were isolated following Seahorse protocols (http://www.seahorsebio.com /resources/tech-writing/techbrief-iso-mito.pdf). In brief, fresh livers were rinsed several times to remove blood, minced in 10 volumes of MSHE+BSA buffer and disrupted using a dounce homogenizer (2-3 strokes). Everything was performed on ice. Homogenates were centrifuged at 8,000 × *g* for 10 minutes at 4ºC, fat/lipid layer was carefully aspirated and supernatant was centrifuged again at 8,000 × *g* for 10 minutes at 4ºC. Pellet was resuspended in MSHE+BSA and recentrifuged. Final pellet was resuspended in minimal volume of MSHE+BSA and mitochondria were quantified by the BioRad protein assay. Mitochondria were diluted 1/10 in MAS1X buffer with the corresponding substrates, seeded in XF24 cell culture microplates (Seahorse Bioscience) at 5 µg in 50 µl per well and centrifuged at 2,000 × *g* for 20 minutes at 4ºC.

Complex I respiration measurements were made in the presence of glutamate (10 mM) and malate (2 mM), followed by the addition of, ADP (4 mM) (state 3 respiration). Complex II respiration measurements were made in the presence of glutamate (10 mM), malate (10 mM), succinate (10 mM) and rotenone (2 µM), followed by the addition of, ADP (4 mM) (state 3 respiration).

Table 4.7. Incubation conditions, dilution, supplier and catalog number for each antibody employed in this study for Western blotting.

Antibody	Supplier	Dilution	Incubation solution	Catalog No.
Akt	Cell Signaling	1:1000	TBS-Tween (0.1%)-BSA (5%)	9272S
AMPKα1	Upstate	1:1000	TBS-Tween (0.1%)-BSA (5%)	07-350
p-AMPKα1 (Thr172)	Cell Signaling	1:1000	TBS-Tween (0.1%)-BSA (5%)	2531S
c-Jun	Cell Signaling	1:1000	TBS-Tween (0.1%)-milk (5%)	9165S
COXIV	Cell Signaling	1:1000	TBS-Tween (0.1%)-milk (5%)	4850S
FLAG	Sigma-Aldrich	1:1000	TBS-Tween (0.1%)-milk (5%)	F1804
GAPDH	Abcam	1:5000	TBS-Tween (0.1%)-milk (5%)	ab8245
Histone H3	Abcam	1:1000	TBS-Tween (0.1%)-BSA (5%)	ab1791
Acetyl-Histone H3	Millipore	1:1000	TBS-Tween (0.1%)-BSA (5%)	06-599
HA	Covance	1:1000	TBS-Tween (0.1%)	MMS-101R
HDAC4	Proteintech	1:1000	TBS-Tween (0.1%)-milk (5%)	17449-1-AP
HuR	Santa Cruz	1:5000	TBS-Tween (0.1%)-milk (5%)	SC-5261
JNK	Cell Signaling	1:1000	TBS-Tween (0.1%)-milk (5%)	9252S
pJNK (Thr183/Tyr185)	Invitrogen	1:1000	TBS-Tween (0.1%)-milk (5%)	44682G
LKB1	Santa Cruz	1:1000	TBS-Tween (0.1%)-milk (5%)	SC-32245
MCJ	*Provided by Dr. Rincon*	1:500	TBS-Tween (0.1%)-milk (5%)	-
Mdm2	Calbiochem	1:1000	TBS-Tween (0.1%)-milk (5%)	OP145
NDUFA9	Invitrogen	1:1000	TBS-Tween (0.1%)-milk (5%)	459100
Nedd8	Abcam	1:1000	TBS-Tween (0.1%)-milk (5%)	Y297
NRF2	Santa Cruz	1:1000	TBS-Tween (0.1%)-milk (5%)	SC-722
p21	Santa Cruz	1:1000	TBS-Tween (0.1%)-milk (5%)	SC-397
p27	Santa Cruz	1:1000	TBS-Tween (0.1%)-milk (5%)	SC-528
PARP	Cell Signaling	1:1000	TBS-Tween (0.1%)-milk (5%)	#9542
PHB1	Santa Cruz	1:1000	TBS-Tween (0.1%)-milk (5%)	SC-28259
SDHA	Abcam	1:5000	TBS-Tween (0.1%)-milk (5%)	ab14715
Ubiquitin	Santa Cruz	1:1000	TBS-Tween (0.1%)-milk (5%)	SC-8017
V5	Invitrogen	1:1000	TBS-Tween (0.1%)-milk (5%)	R96025
β-Actin	Sigma-Aldrich	1:5000	TBS-Tween (0.1%)-milk (5%)	A5441
Anti-mouse IgG, HRP linked antibody	Cell Signaling	1:5000	TBS-Tween (0.1%)-milk (5%)	#7076
Anti-rabbit IgG, HRP linked antibody	Cell Signaling	1:5000	TBS-Tween (0.1%)-milk (5%)	#7074

APAP 10mM was injected and the inhibition ratio of complexes I and II by APAP was determined. The production of ATP through complex I and II was calculated by the addition of oligomycin (1 mM).

For normal OCR measurements mitochondria were incubated in the presence of substrates for both complexes I and II: glutamate (10 mM), malate (2mM) and succinate (10 mM). After an OCR baseline measurement, sequential injections through ports in the XF Assay cartridges of pharmacologic inhibitors: ADP (4 mM) (state 3 respiration), oligomycin (3 μM), FCCP (4 μM) and finally rotenone (2 μM) and antimycin A (4 μM), mitochondrial complex I and III inhibitors respectively, were performed and changes in OCR were analyzed. The normalized data were expressed as pmol of O_2 per min or milli-pH units (mpH) per min, per viability measured by MTT assay.

4.7 ATP MEASUREMENT

ATP levels were evaluated by two different methods:

ATP-coupled oxygen consumption through OXPHOS was measured using the Seahorse Bioscience XF24-3 Extracellular Flux Analyzer and the ATP synthase inhibitor oligomycin, as described in section 4.6.

Intracellular ATP levels in primary hepatocytes or liver mitochondria were determined using the

ATPlite™ luminescence ATP detection kit (Perkin Elmer) by following the recommendations from the manufacturer. Final values were normalized to total protein concentration.

4.8 ROS MEASUREMENTS

4.8.1 Oxidative stress

Oxidative stress was measured using CellROX™ Deep Red reagent (Life Technologies), following manufacturer's instructions. Briefly, primary hepatocytes were incubated in 1 ml of growth medium containing CellROX™ Deep Red 5 µM in a CO_2 incubator at 37ºC for 30 minutes. After, cells were washed twice with PBS, trypsinized and run on a flow cytometer for fluorescence analysis at an excitation of 640 nm and emission of 665 nm.

4.8.2 Mitochondrial ROS

mROS was measured using MitoSOX™ Red reagent (Life Technologies), following manufacturer's instructions. Briefly, primary hepatocytes were incubated in 1 ml of growth medium containing MitoSOX 1.5 µM in a CO_2 incubator at 37ºC for 10 minutes in the same 12-multiwell plate where they had been seeded. After, cells were washed twice with PBS and the fluorescence was measured using a spectrophotometer at an excitation of 510 nm and emission of 595 nm. Final values were normalized to total protein concentration.

4.9 MMP MEASUREMENT

MMP was measured using JC-1 dye (Life Technologies), following manufacturer's instructions. Primary hepatocytes were incubated in 1 ml of growth medium containing JC-1 dye at 10 µg/µl for 10 minutes at 37ºC protected from light. After, cells were washed twice with PBS, trypsinized and run on a flow cytometer for fluorescence analysis at an excitation of 514 nm and emission of 529/590 nm. Fluorescence emission shifts from green (~529 nm) to red (~590 nm) when JC-1 exhibits a potential-dependent accumulation in mitochondria. Consequently, mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio.

4.10 CELL DEATH MEASUREMENTS

4.10.1 Caspase-3 activity assay

Caspase-3 activity was assayed by using a fluorescent substrate (Ac-DEVD-AFC) (ALX-260-032-M001 Enzo). Cultured cells and frozen livers were homogenized in ice-cold lysis buffer without protease inhibitors (Hepes 10mM pH 7.4, EDTA 2mM, Chaps 0.1% and DTT 5mM M) and centrifuged for eliminating the debris. For each sample, a 500 µl mix containing 10 to 50 µg of protein, 20 µl of 25x reaction buffer (Pipes 250 mM pH 7.4, EDTA 50 mM, Chaps 2.5% and DTT 125 mM) and 2.5 µl of Ac-DEVD-AMC was prepared. This reaction mixture was divided into two duplicates of 200 µl and placed onto a UV Flat Bottom 96-well microtiter plate (Thermo). Kinetic of enzymatic activity was measured at 0, 1 and 2 hours in a Spectramax M3 spectrophotometer (Molecular Devices) at an excitation of 390 nm and emission of 510 nm. The microplate with the samples was kept at 37ºC, protected from light and shaking during the assay.

4.10.2 TUNEL assay

Cell death was analyzed in primary hepatocytes seeded in rat collagen type I-coated coverslips by using TUNEL (In situ Cell Death detection Kit; Roche) according to manufacturer's instructions. Basically, coverslips were fixed with ice-cold 4% paraformaldehyde in PBS for 10 minutes, washed twice with PBS, incubated with a H2O2 (30%)/MeOH (3%) solution for 5 minutes, then with sodium citrate for 2-4 minutes and finally, with a mix containing TUNEL enzyme and diluent buffer (dilution 1/50) overnight at 4ºC or 1 hour at 37ºC. Mounting solution containing 4,6-diamidino-2-phenylindole (DAPI) was used to counterstain the nuclei of hepatocytes. At least 5 images of each coverslip were taken and positive nuclei were measured using ImageJ64 software (NIH).

4.11 PROTEIN STABILITY ASSAY

Primary hepatocytes were transfected with control or Nedd8 siRNA (siNedd8) as described in section 4.3.3. After, cycloheximide (CHX; 50 µg/mL) was added and cells were lysed at the indicated time points. Protein stability was analyzed by Western blotting using the indicated antibodies, quantified with Image J software, and presented as the percentage of remaining protein. Data are representative from three independent experiments.

4.12 ENZYMATIC ASSAYS

4.12.1 MDH2 activity

MDH2 activity was measured in mouse livers using the Mitochondrial Malate Dehydrogenase Activity Assay Kit (Abcam), following manufacturer's instructions. Liver lysates were prepared by homogenization with PBS using a dounce homogenizer to a final concentration of 25 mg/mL (5 mg of frozen liver were used). Homogenates were solubilized by adding 4 volumes of extraction buffer to a protein concentration of 5 mg/mL and incubated on ice for 20 minutes. After, homogenates were centrifuged at 16,000 × g for 20 minutes at 4ºC and supernatants were transferred into clean tubes. 100 µl of each diluted sample were added per well to the MDH2 antibody coated microplate and were incubated for 3 hours at room temperature. Then, wells were washed twice with washing buffer and 100 µl of fresh mixed 1X Activity Solution were added to each well. Kinetic of enzymatic activity was measured for 15-30 minutes every 20 seconds at 450 nm. Final values were normalized to total protein concentration and the activity was expressed as ΔmOD/min.

4.12.2 G6PDH activity

Frozen livers (40 mg) were homogenized using a Potter homogenizer (20 strokes) with 10 volumes of a buffer containing KCl pH 7.6 150 mM, $MgCl_2$ 1mM, dithiothreitol 0.5 mM and N-acetylcysteine 10 mM. Then, liver homogenate was centrifuged at 105,000 × *g* for 1 hour at 4 °C. The supernatant, corresponding to the cytosolic fraction was used to measure the G6PDH as described[428]. The reaction buffer (glycylglycine pH 8 100 mM, $NADP^+$ 10 mM, $MgSO_4$ 150 mM, G6P 30 mM and NADPH 2.4 mM) was pre-incubated at 37ºC for 5 mins. The activity was assessed in 120 mg of protein from the cytosolic fraction for 5 mins at 37ºC in agitation. The reaction was stopped introducing the samples on ice. Finally, the NADPH that was produced was measured at 340 nm. The activity was expressed as nmol of produced NADPH per mg of protein and mins.

4.12.3 PEMT activity

1,000,000 primary hepatocytes and 200,000 BCLC3 and BCLC5 cells were seeded in 60 mm culture plates. MLN4924 treatment is described in section 4.3.2 and Nedd8 knockdown in section 4.3.3. Cells were incubated with [^{3}H] ethanolamine (5 µCi/ml). Then, cells and medium were separated, lipids extracted, separated, and the label incorporated into PE and PC determined in a scintillation counter.

4.13 UPS MEASUREMENTS

4.13.1 Proteasome activity assay

For the *in vitro* assay of 26S proteasome activities, liver samples were collected in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 and 2 mM ATP) without protease inhibitors. Lysate was centrifuged at 10,000 × *g* for 10 min at 4 °C. Approximately 15–25 µg of total protein of cell lysates were collected in proteasome activity assay buffer (250 mM HEPES, pH 7.5, 5 mM EDTA, 0.5% NP40, 0.01% SDS and 2 mM ATP) and were transferred to a UV Flat Bottom 96-well microtiter plate (Thermo) with 20µM of the fluorogenic substrate Suc-Leu-Leu-Val-Tyr-AMC (Enzo BML-P802-0005) to measure the caspase-like activity of the proteasome. Free AMC liberated by the substrate hydrolysis was quantified for 2-3 h at 37 °C. Fluorescence (380 nm excitation, 460 nm emission) was monitored using a SpectraMax M2 plate reader (Molecular Devices, CA, USA). Preliminary experiments with control cells indicated that reaction rates were linear for at least 4 hours. The data were plotted as Relative Fluorescence Units (RFU).

4.13.2 Isolation of ubiquitylated proteins using TUBEs

Total ubiquitylated proteins were extracted from murine livers using GST tandem ubiquitin-binding entities (TUBEs). Frozen livers were homogenized in lysis buffer (20 mM Tris–HCl, pH 8, 2 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT. Add extemporary 1 mM pefabloc SC and one mini-tablet of complete protease inhibitor cocktail (Roche)) (1 ml per 75 mg of tissue), including 100 µg of TUBES. TUBEs were produced in *Escherichia coli* (C41-DE3) using a standard protocol for the production of recombinant proteins[429]. Then, lysates were clarified by centrifugation at 15500 × *g* and 4°C for 10 min. Supernatant was collected and a total volume of 1/10 was taken and diluted in equal volume of 3x boiling buffer. This fraction is considered as input. 450 µl of clarified lysate were added to 100 µl glutathione beads slurry. Incubate lysate with beads, slowly rotating at 4°C for at least 2 h (slow binding reaction). Beads were spin down (300 × *g* for 5 min) and supernatant was collected for analysis. TUBES were washed three times with 1 ml ice-cold PBS, 0.05% Tween 20 and spin down at 4°C and 300 ×*g* for 1 min and all liquid was aspirated carefully. The beads correspond to TUBEs BOUND. Sample was eluted with 30 µl 1:1 3x boiling buffer and lysis buffer (50 mM NaF (Sigma), 5 mM tetra-sodium pyrophosphate (Sigma), 10 mM β-glyceropyrophosphate (Sigma), 0.2% Igepal, 2 mM EDTA, 20 mM Na_2HPO_4, 20 mM NaH_2PO_4, 1 mM Pefabloc SC, and one mini-tablet of complete protease inhibitor cocktail (Roche)). Pull-down material was analyzed by Western blot with a specific antibody against HDAC4.

4.14 PROTEIN IMMUNOPRECIPIATION ASSAY

Protein-protein complexes were immunoprecipiatated as follows:

Cell lysates preparation: primary WT and *Phb1* KO hepatocytes untreated or treated with DCA were lysed in Nonidet P-40 (NP40) buffer (Tris-HCl pH 8.5 50 mM, NaCl 150 mM, NP-40 1%, EDTA 5 mM) supplemented with protease and phosphatase inhibitors cocktail (Roche). Whole-cell lysates were processed and quantified for protein content as described in section 4.5.1.

Covalent cross-linking of antibodies to beads: In order to limit the recovery of light and heavy antibody chains during immunoprecipitation, antibodies were covalently cross-linked to Protein A-Sheparose beads (Sigma-Aldrich). 100 µl of Protein A-Sheparose beads per sample were washed four times in PBS (5000 rpm, 5 minutes at 4 ºC) and incubated overnight with 10 µg of the appropriate primary antibody: PHB1 (Cell Signaling) and IgG2a (BD Pharmingen) as negative control. After incubation, beads were washed three times (2500 rpm, 5 minutes at 4ºC) with NaBorate buffer (Borate 0.2 M, NaCl 3M, pH 9), and covalently cross-linked with NaBorate buffer containing dimethyl pimelimidate for 30 minutes at room temperature with agitation. Beads were then washed three times with NaBorate buffer and incubated with ethanolamine (pH 8 0.2 M) for 2 hours at room temperature with agitation. Covalent cross-link reaction was stopped by washing beads three times with fresh glycine buffer (pH 2.5 200 mM). Beads were washed three times with PBS and kept at 4ºC until the incubation with the protein extract.

Immunoprecipiation assay: covalently cross-linked beads were incubated with 500 µg of protein lysate overnight at 4ºC with agitation. After incubation, beads were washed three times with NP-40 lysis buffer and bound proteins were eluted by heating at 95ºC for 5 minutes in 2x SDS-PAGE sample buffer. Immunoprecipitated proteins (IP) and original cell extracts (INPUT) were analyzed by Western blotting with the appropriate antibodies.

4.15 PROTEIN-HISTIDINE AFFINITY PURIFICATION USING NICKEL-NITRIOLOTRIACETIC ACID (Ni^{2+}-NTA) BEADS

Primary hepatocytes were cotransfected with His_6-Nedd8 and FLAG-LKB1 or HA-Akt constructs as described in section 4.3.2. Cells were lysed in guanidinium-HCl 6 M, Na_2HPO_4/NaH_2PO_4 0.1 M, Tris-HCl pH 8 0.01 M, plus β-mercaptoethanol 10 mM and imidazole 5mM. Lysates were mixed with 70 µl of low density Ni^{2+}-NTA-agarose beads precoated with BSA and prewashed with lysis buffer. Lysates were incubated with the beads for 3 hours at room temperature, successively washed first with lysis buffer, then twice with urea 8 M, Na_2HPO_4/NaH_2PO_4 0.1 M, Tris-HCl pH 8 0.01 M plus β-mercaptoethanol 10 mM, and finally thrice with urea 8 M, Na_2HPO_4/NaH_2PO_4 0.1 M, Tris-HCl pH 6.3 0.01 M plus β-mercaptoethanol 10 mM. After the last wash, the beads were eluted with imidazole 200 mM in SDS 5%, Tris-HCl pH 6.7 0.15 M, glycerol 30%, β-mercaptoethanol 0.72 M. The eluates were subjected to SDS-PAGE and the proteins transferred to a nitrocellulose membrane for Western blotting.

4.16 QUANTIFICATION OF HEPATIC LIPIDS

Liver: 30 mg of frozen livers were homogenized with 10 volumes of ice-cold phosphate buffered saline (PBS) in a Potter homogenizer (20 strokes). FAs were measured in homogenates using a kit from Wako Chemicals (Richmond, VA) and lipids were quantified as described[430]. Briefly, lipids were extracted from 1.5 mg of protein from liver homogenates[431]. PC, PE, DG, FC and CE were separated by thin layer chromatography and quantified as described[432]. TGs were measured in the lipid extract with a kit from A. Menarini Diagnostics (Italy).

Primary hepatocytes: Hepatocytes seeded in rat collagen type I-coated coverslips were incubated with BODIPY 493/503 (Molecular Probes) at a concentration of 1 mg/ml during 30 min prior to fixation (ice-cold paraformaldehyde 4% in PBS, 10 minutes). Quantification of lipid bodies was performed using Frida Software.

4.17 HEPATIC DNL

DNL was performed as previously described[433] with slight modifications. In brief, freshly isolated tissue slices (40 mg) were incubated in high glucose DMEM with insulin (150nM) and [H^3] Acetic acid 20 µCi/ml for 4 hours. Tissue slices were washed five times in cold PBS, homogenated in PBS and lipids were extracted as previously described[431]. Then, lipids were separated by TLC[432], each lipid was scraped and the radioactivity was measured in a scintillation counter.

4.18 β-OXIDATION

β-oxidation was assessed as described before[434,435]. Fresh liver pieces were homogenated in a Potter homogenizer (5 strokes) in cold buffer (Tris-HCl 25mM, sucrose 500nM, EDTA-Na_2 pH 7.4 1mM) and sonicated for 10 seconds. Then, the homogenates were centrifuged at 500 × *g* for 10 mins at 4°C. Approximately 500µg of protein from the homogenates supernatant was used for the assay in a volume of 200µl. The reaction started by adding 400µl of assay mixture containing 0.5 µCi/ml [1-^{14}C] palmitic acid to the samples and was incubated for 1 hour at 37°C in eppendorf tubes with a Whatman paper circle in the cap. The reaction was stopped by adding 300µl of perchloric acid 3M and NaOH 1M was added to impregnate the Whatman cap. After 2 hours, the Whatman caps were retired and the radioactivity associated was measured in a scillation counter. Eppendorf tubes were centrifugated at 2100 × *g* 10 mins at 4°C. 400µl from the supernatant were collected and the radioactivity was counted in a scillation counter. The supernatant contained the acid-soluble metabolites (ASM) and the Whatman caps captured the released CO_2.

4.19 GSH AND NAPQI-GSH CONTENT

Liver extracts were analyzed with a UPLC system (Acquity, Waters, Manchester) coupled to a Time of Flight mass spectrometer (TOF MS, SYNAPT G2, Waters). A 2.1 x 100 mm, 1.7 µm BEH amide column (Waters), thermostated at 40 °C, was used to separate the analytes before entering the MS. Solvent A (aqueous phase) consisted of 99.5% water, 0.5% formic acid and 20 mM ammonium formate while solvent B (organic phase) consisted of 29.5% water, 70% MeCN, 0.5% formic acid and 1 mM ammonium formate. The extracted ion traces were obtained for GSH (m/z = 308.0916) and NAPQI-GSH (m/z = 457.139) in 20 mDa windows and subsequently smoothed (2 points, 2 iterations) and integrated with QuanLynx software (Waters, Manchester). Concentrations were converted into amount of analyte per mg liver tissue.

4.20 ELISA

TNFα cytokine levels in liver extracts were determined by using the DuoSet ELISA Development Kit according to the manufacturer's instructions (R&D Systems, Minnesota, USA).

4.21 HISTOLOGICAL TECHNIQUES

4.21.1 Histology

Hematoxylin & eosin: Paraffin-embedded sections (5 µm thick) of formalin-fixed liver samples were deparaffinized in Histo-Clear and rehydrated

through graded alcohol solutions. Once hydrated, sections were placed in hematoxylin solution for 5 minutes and washed in running tap water for 5 minutes. Then, sections were placed in eosin solution for 15 minutes, washed in running tap water for 3 minutes, dehydrated, cleared and mounted using DPX mounting medium.

PAS: Paraffin-embedded sections (5 µm thick) of formalin-fixed liver samples were deparaffinized in xylene and rehydrated through graded alcohol solutions. Once hydrated, sections were oxidized in 1% periodic acid solution for 10 minutes, rinsed in distilled water and placed in Schiff reagent for 20 minutes. After, sections were washed in running tap water for 5 minutes and counterstain with hematoxylin for 1 minute. Sections were washed in tap water for 5 minutes, dehydrated and mounted using DPX mounting medium.

Siruis red: Paraffin-embedded sections (5 µm thick) of formalin-fixed liver samples were deparaffinized in xylene and rehydrated through graded alcohol solutions. Once hydrated, sections were placed in 0.01% Fast Green FCF picric acid solution for 15 minutes, in 0.04% Fast Green FCF/0.1% sirius red picric acid solution for 15 minutes, dehydrated and mounted using DPX mounting medium.

Sudan III: Frozen liver tissue sections (8-10 µm thick) were air dried for 2 minutes, fixed in formalin for 2 minutes, washed with running tap water for 1 minute, rinsed with 60% isopropanol, stained with freshly prepared Sudan III working solution (freshly prepared in darkness) for 30 minutes and rinsed with 60% propanol. After, sections were lightly counterstained with hematoxylin, rinsed with distilled water and mounted in aqueous mountant.

4.21.2 IHC

Sections were unmasked according to the primary antibody to be used and subjected to peroxide block (3% H_2O_2 in 1x PBS) for 10 minutes at RT. For stainings with mouse-hosted primary antibodies in mouse tissues, samples were blocked with goat anti-mouse Fab fragment (Jackson Immunoresearch) (1 hour, RT, 1:10) before being blocked with serum (5% goat serum in 1X PBS) for 30 minutes at RT. Then, sections were incubated in a humid chamber with primary antibody in DAKO antibody diluent (DAKO) followed by Envision anti-rabbit or anti-mouse (DAKO) or ImmPRESS anti-rat (Vector) HRP-conjugated secondary antibody incubation for 30 minutes at RT. Unmasking and incubation conditions for each antibody are shown in Table 4.8. Colorimetric detection was performed with Vector VIP (purple) chromogen (Vector) and sections were counterstained with hematoxylin. Samples were dehydrated through graded alcohol solutions and Histo-Clear and mounted using DPX mounting medium. For αSMA staining, sections were incubated with primary antibody, which is conjugated to Cy3, and mounted with Fluoromount-G (Southern Biotech.) containing 0.7 mg/l of DAPI. For the analysis, 10 images per liver sample with a 40x objective from upright light microscope (Carl Zeiss AG). Fluorescence was examined on a Zeiss Fluorescence microscope using 40x objective. The average sum of intensities and stained area percentage of each patient/sample was calculated using FRIDA software (http://bui3.win.ad.jhu.edu/frida/, Johns Hopkins University).

4.21.3 TUNEL assay

Necrosis was analyzed by using TUNEL (In situ Cell Death detection Kit; Roche) according to the manufacturer's instructions. Paraffin-embedded sections (5 µm thick) of formalin-fixed liver samples were treated with proteinase K for 15 minutes at RT and subjected to peroxide block (3% H_2O_2 in methanol) before incubation with a mix containing TUNEL enzyme and diluent buffer (dilution 1/50) for 2 hours at 37°C. Sections were mounted using Fluoromount-G (Southern Biotech.) containing 0.7 mg/l of DAPI. Fluorescence was examined a Zeiss Fluorescence microscope using 40x objective.

4.22 GLUCOSE, KB AND TGS IN SERUM

Glucose was measured with a glucometer (Arkray Factory, Japan) in blood obtained from the tail vein. KB were measured using a commercially available kit from Wako Chemicals (Richmond, VA). TGs were measured using a commercially available kit Triglycerides Liquid Mono (Krotest laboratorios).

4.23 METABOLOMIC ANALYSIS

UPLC®-MS metabolomics analysis was performed in livers from untreated *Phb1* KO and MLN4924 treated mice. Briefly, four UPLC®/TOF-MS based platforms analyzing methanol; methanol/water and chloroform/methanol liver extracts were combined. Identified ion features in the methanol extract platform included fatty acyls, BAs, and lysoglycerophospholipids. The extracts prepared for methanol platform were also derivatized for amino acid analysis. The chloroform/methanol extract platform provided coverage over glycerolipids, CEs, sphingolipids and glycerophospholipids. Finally, the methanol/water extract platform comprised the study of polar metabolites, such as vitamins, nucleosides, nucleotides, carboxylic acids, coenzyme A derivatives, carbohydrate precursors/derivatives and redox-electron-carriers. For this platform, a mixture of methanol/water (60:40, v/v) containing non-endogenous internal standards was added to liver tissue (50:1, v/w) and homogenized using a Precellys 24 grinder. After 1 hour of incubation at -20ºC samples were centrifuged at 16,000 × g for 15 mins. The supernatant was collected and chloroform was added. Polar phase was then transferred to a clean tube for solvent evaporation. Dried extracts were resuspended in water and, after centrifugation; supernatants were transferred to vials for UPLC®-MS analysis. Lipid nomenclature follows the LIPID MAPS convention, www.lipidmaps.org.

Data obtained with the UPLC®-MS were processed with the TargetLynx application manager for MassLynx (Waters Corp.). All the calculations were performed with R v2.13.0 (R Development Core Team, 2010).

4.24 PREDICTIVE MODELING OF HCC OUTCOME USING NEDD8, LKB1 AND AKT SIGNATURE

Logistic regression was performed to quantify the predictability of Nedd8, LKB1 and Akt model. In the absence of an independent set, we evaluated the performance of the model using leave- one-out cross-validation. ROC-related computation was performed using DiagnosisMed (http://CRAN.R-project.org/ package _ Diagnosis Med) and pROC package. All computations were performed using R software (http://expasy.org/tools/ pROC).

4.25 STATISTICAL ANALYSIS

All experiments were performed in triplicate with data expressed as means ± SEM (standard error of the mean). Statistical significance was estimated with Student's test. For IHC analysis in human samples, logistic regression and Pearson's correlation coefficient were calculated by SPSS program. A p value < 0.05 was considered significant. Liver metabolite concentrations were compared using unpaired Student´s or Welch´s t test where unequal variances were found.

Table 4.8 Unmasking and incubation conditions, dilution, supplier and catalog number for each antibody employed in this study for IHC.

Antibody	Supplier	Incubation conditions	Unmasking conditions	Catalog No.
AFP	Abcam	1.200 Overnight at 4ºC	Citrate buffer pH 6.0 (20 min at 97ºC)	ab6799
Akt	Abcam	1.25 (Human)/1.100(Mouse) Overnight at 4ºC	Citrate buffer pH 6.0 (20 min at 97ºC)	ab32505
CK19	Hybridoma Bank	1.200 1h at RT	EDTA pH 8.0 (20 min at 37ºC)	TROMA-III-s
F4/80	Bio RAD	1.50 1h at 37ºC	Proteinase K (15 min at RT	MCA497BB
HDAC1	Cell Signaling	1.100 Overnight at 4ºC	Citrate buffer pH 6.0 (20 min at 97ºC)	5356
HDAC2	Cell Signaling	1.100 Overnight at 4ºC	Citrate buffer pH 6.0 (20 min at 97ºC)	5113
HDAC3	Cell Signaling	1.100 Overnight at 4ºC	EDTA pH 8.0 (20 min at 600w)	2632
HDAC4	Proteintech	1.100 Overnight at 4ºC	Citrate buffer pH 6.0 (20 min at 600W)	17449-1-AP
HDAC5	Cell Signaling	1.50 Overnight at 4ºC	Citrate buffer pH 6.0 (20 min at 600W)	2082
HuR	Santa Cruz	1.100 1h at RT	Citrate buffer pH 6.0 (20 min at 97ºC)	SC-5261
LKB1	Abcam	1.50 Overnight at 4ºC	Citrate buffer pH 6.0 (20 min at 97ºC)	ab58786
MCJ	BioMosaics	1.100 Overnight at 4ºC	Citrate buffer pH 6.0 (20 min at 97ºC)	B0027R
NAE1	Abgent	1.100 Overnight at 4ºC	Citrate buffer pH 6.0 (20 min at 97ºC)	API3067C
Nedd8	Cell Signaling	1.300 Overnight at 4ºC	Citrate buffer pH 6.0 (20 min at 97ºC)	2745S
PHB1	Cell Signaling	1.100 Overnight at 4ºC	Citric acid pH 6.0 (30 min at 600W)	2426S
Smad2/3	Cell Signaling	1.50 Overnight at 4ºC	Citrate buffer pH 6.0 (20 min at 600W)	3102S
Ubiquitin	Sigma	1.250 Overnight at 4ºC	Tris-EDTA pH 9.0 (5 min at 900W + 15 min at 600w	U5379
αSMA	Sigma	1.200 Overnight at 4ºC	Citrate buffer pH 6.0 (20 min at 600W)	C6198

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